

# Oestrogen and progesterone increase the levels of apoptosis induced by the human papillomavirus type 16 E2 and E7 proteins

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Human papillomavirus (HPV) type 16 infects the genital tract and is generally acknowledged to be a causative agent of cervical cancer. HPV infection alone is not sufficient to induce cervical cancer and other factors such as steroid hormones are thought to play a role in the establishment and/or progression of this disease. The HPV-16 E2 protein is required for virus replication and modulates viral gene expression whereas the HPV-16 E7 protein is required for cell transformation. We and others have shown that both the E2 and E7 proteins can induce apoptotic cell death in HPV-transformed and non-HPV transformed cell lines. Here we show that the steroid hormones oestrogen and progesterone can both increase the levels of E2- and E7-induced apoptosis. The oestrogen metabolite 16 $\alpha$ -hydroxyoestrone also increases E2- and E7-induced cell death and the dietary component indole-3-carbinol, which reduces the formation of 16 $\alpha$ -hydroxyoestrone from oestrogen, blocks the effects of oestrogen. Thus the metabolism of oestrogen to 16 $\alpha$ -hydroxyoestrone appears to be required for the effects of this hormone on E2- and E7-induced cell death. We also show that the oestrogen receptor antagonist 3-hydroxytamoxifen blocks the effects of oestrogen on E2- and E7-induced cell death, whereas the anti-progesterone RU486 blocks the effects of both progesterone and oestrogen. We discuss these results in terms of the origin and progression of cervical cancer.

## Introduction

Several types of human papillomavirus (HPV) are associated with cervical cancer (reviewed by van Ranst *et al.*, 1996). HPV-16 is the most common viral genotype found in malignant cervical lesions and probably accounts for around half of all cases of this disease (Bosch *et al.*, 1995; Walboomers *et al.*, 1999). However, cervical cancers do not produce viral particles and are thus not thought to represent the typical outcome of an HPV-16 infection. In HPV-infected cells, the HPV genome exists as an extrachromosomal DNA circle. In contrast, in HPV-transformed cells from cervical tumours, the viral genome is often integrated into the host genome (Dürst *et al.*, 1985). Integration frequently occurs within the viral E2 gene and this results in loss of the E2 gene product (Baker *et al.*, 1987; Corden *et al.*, 1999). These observations suggest that the absence of E2 protein is a major factor in the development of cervical cancer (Schneider-Maunoury *et al.*, 1987).

The HPV E2 proteins are required for efficient virus replication and are thought to play a role in the regulation of HPV gene expression (reviewed by Thierry, 1996). More recently, the E2 proteins have also been shown to have dramatic effects on cell survival. The HPV-16 E2 protein can induce apoptotic cell death in a variety of HPV-transformed and non-HPV-transformed cell lines (Sanchez-Perez *et al.*, 1997; Webster *et al.*, 2000). Similarly, the E2 proteins from HPV-18 and HPV-33 have been shown to induce apoptosis in HeLa cells, an HPV-18-transformed cell line, and in normal human foreskin keratinocytes, respectively (Desaintes *et al.*, 1997; Frattini *et al.*, 1997). The bovine papillomavirus E2 protein has been shown to induce growth arrest in HeLa cells by down-regulating expression of the integrated HPV-18 oncogenes (Francis *et al.*, 2000). However, we have shown that the DNA-binding activity of the HPV-16 E2 protein is not required for induction of apoptosis in these cells (Webster *et al.*, 2000). Thus, the ability of the E2 proteins to induce cell death is not purely a consequence of their ability to bind DNA and alter viral gene expression. Taken together these observations suggest that following HPV-16 infection, the E2 protein could generate a pro-apoptotic signal within the cell.

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The HPV-16 E7 protein brings about cell proliferation and can also induce apoptosis (reviewed by Crook & Vousden, 1996). E7 binds to the Rb tumour suppressor protein and the Rb-related proteins p107 and p130 (Dyson *et al.*, 1989). The binding of E7 to Rb–E2F complexes brings about the release of E2F and targets Rb for ubiquitin-dependent proteolysis (Boyer *et al.*, 1996; Jones *et al.*, 1997). Once released from Rb, E2F-1 activates the transcription of genes required for S-phase and can also induce apoptosis (Wu & Levine, 1994; Qin *et al.*, 1994; Field *et al.*, 1996). Overexpression of the HPV-16 E6 protein can block both E2- and E7-induced apoptosis (Webster *et al.*, 2000). One function of the E6 protein is to bind the p53 tumour suppressor protein and target this protein for degradation (Scheffner *et al.*, 1990; Werness *et al.*, 1990; Lechner *et al.*, 1992; Hubbert *et al.*, 1992). Interestingly, the HPV-16 E2 protein can interact physically with p53 and both E2 and E7 are capable of inducing p53-dependent apoptosis (Massimi *et al.*, 1999; Webster *et al.*, 2000). Thus, during virus infection the pro-apoptotic signals generated by E7 and E2 are probably counter-balanced by the E6 protein. This is in agreement with studies that have shown decreased apoptosis and increased cell proliferation in HPV-infected cervical epithelium (Nair *et al.*, 1999). We have suggested previously that random integration events which disrupt the HPV-16 E2 gene upset this balance between pro-apoptotic and anti-apoptotic signals and result in cells that are more likely to proliferate and, therefore, more likely to produce cervical cancer (Sanchez-Perez *et al.*, 1997). Since the E2 protein regulates HPV gene expression, another possibility is that disruption of the E2 gene leads to the deregulation of E6 and E7, which in turn leads to increased cell proliferation (Francis *et al.*, 2000, and references therein).

HPV-16 infection alone is probably insufficient to cause cervical cancer and several possible cofactors have been identified including the steroid hormones progesterone and oestrogen. Progesterone and progestins can act as cofactors in the transformation of baby rat kidney cells by HPV-16 and Ras (Pater *et al.*, 1990). Furthermore, progesterone has been reported to increase HPV-16 and HPV-18 gene expression at the levels of transcription and mRNA stability (Chen *et al.*, 1996; Yuan *et al.*, 1999 *a*; Mittal *et al.*, 1993). Most cases of cervical cancer arise in the most oestrogen-sensitive region of the cervix, an area known as the transformation zone (Autier *et al.*, 1996, and references therein). Furthermore, the incidence of HPV DNA in exfoliated cervical cells increases during pregnancy when oestrogen levels are elevated (Rando *et al.*, 1989; Schneider *et al.*, 1987) and prolonged use of oestrogen-containing oral contraceptives has been reported to double the risk of cervical cancer (Brisson *et al.*, 1994). Although the levels of E7 protein within HPV-transformed cell lines do not appear to change after oestrogen treatment (Selvey *et al.*, 1994), oestrogens have been reported to increase HPV gene expression and orally administered oestrogen can increase the proliferation of cervical cancer cells *in vivo* (Kim *et al.*, 2000; Mitrani-Rosenbaum *et al.*, 1989; Chen *et al.*, 1996;

Bhattacharya *et al.*, 1997). In HPV-18 transgenic mice, HPV gene expression has been shown to vary during pregnancy and to be increased in the presence of oestrogen or progesterone (Michelin *et al.*, 1997). In addition, chronic oestrogen exposure has been shown to induce cervical carcinogenesis in transgenic mice expressing the HPV-16 E6 and E7 genes (Arbeit *et al.*, 1996). Interestingly, in these E6/E7 transgenic mice expression of E6 and E7 is under the control of the human keratin-14 promoter, suggesting that oestrogens do not induce cervical cancer simply by altering HPV gene expression (Arbeit *et al.*, 1996).

Although the mechanism whereby oestrogens act synergistically with E6 and/or E7 to induce cervical cancer is not known, one possibility is that oestrogens might act as survival factors, enabling the enhanced proliferation of cells expressing E6 and E7. This could in turn result in a larger pool of HPV-infected cells within which tumour cells could arise spontaneously. Another possibility is that high levels of DNA damage brought about by the oestrogen metabolite 16 $\alpha$ -hydroxyoestrone might result in the accumulation of mutations that eventually lead to carcinogenesis (Auborn *et al.*, 1991; Newfield *et al.*, 1998). Oestrogen is metabolized extensively within the body to produce a family of related compounds, including 16 $\alpha$ -hydroxyoestrone. 16 $\alpha$ -hydroxyoestrone is highly oestrogenic and has also been shown to be tumorigenic in mice (Swanek & Fishman, 1988; Telang *et al.*, 1992). The transformation zone of the cervix displays a constitutively high level of conversion of oestradiol to 16 $\alpha$ -hydroxyoestrone and when these cells are immortalized by HPV-16 DNA, this activity increases around 8-fold (Auborn *et al.*, 1991).

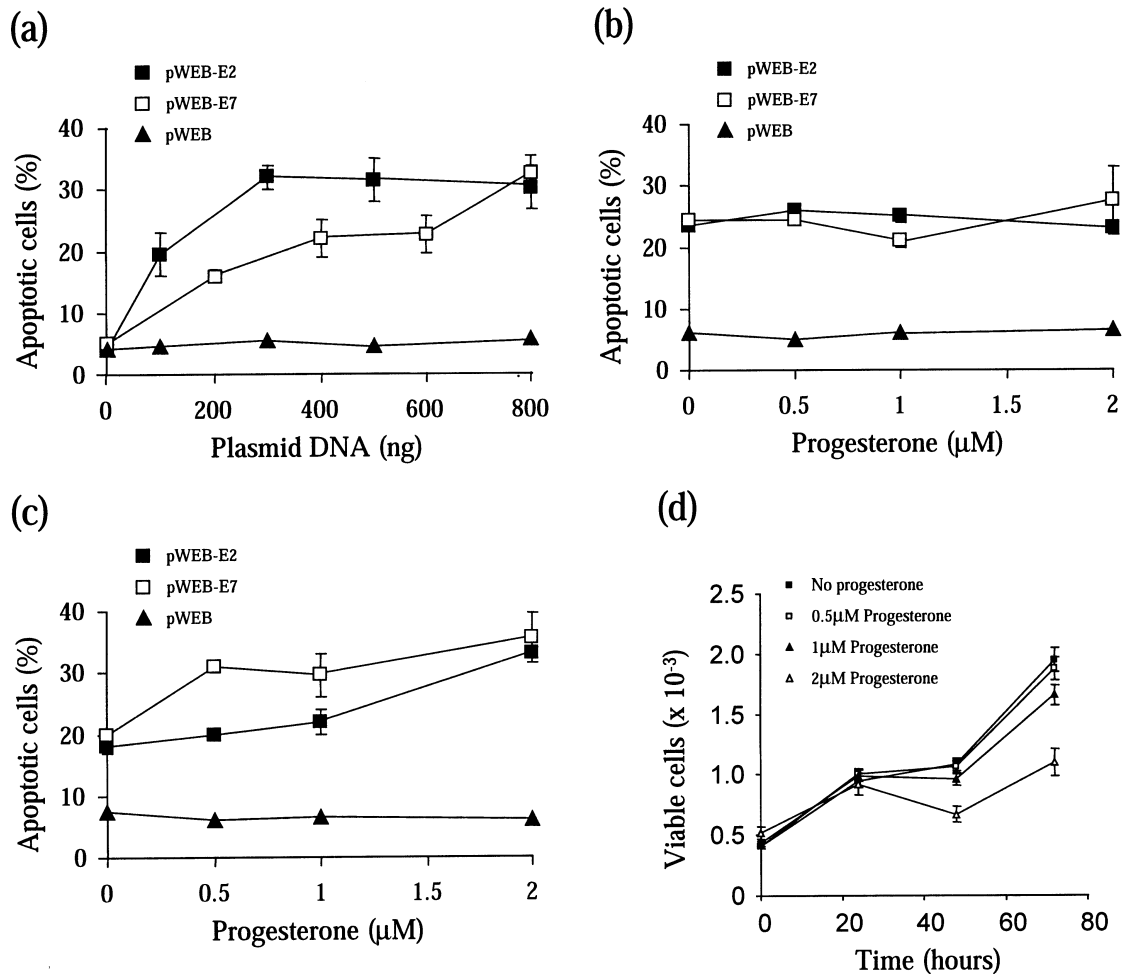
Here we show that progesterone, oestrogen and 16 $\alpha$ -hydroxyoestrone increase the levels of apoptosis induced by the E2 and E7 proteins. We discuss the implications of these findings in terms of the biological roles of the E2 and E7 proteins in HPV-infected cells and in cervical carcinogenesis.

## Methods

■ **DNAs used in this study.** All of the DNAs used in this study have been described previously. The plasmids pWEB-E2 and pWEB-E7 express the HPV-16 E2 and E7 proteins respectively under the control of the CMV promoter (Webster *et al.*, 2000). The plasmid pCMX-GFP3 expresses the green fluorescent protein (GFP) and was kindly supplied by Jeremy Tavaré (Department of Biochemistry, University of Bristol, UK).

■ **Cell culture and transfections.** HeLa cells were maintained in Minimal Essential Medium (MEM; Sigma) supplemented with 10% foetal bovine serum (FBS; Sigma) and penicillin (100 000 U/l) and streptomycin (100 mg/l) at 37 °C in 5% CO<sub>2</sub>. In some cases supplements were added to the media: progesterone (Sigma), RU486 (Sigma), 17- $\beta$ -oestradiol (RBI), 3-hydroxytamoxifen (RBI), indole-3-carbinol (Sigma) and 16 $\alpha$ -hydroxyoestrone (Sigma), at the concentrations stated in the figure legends.

Prior to transient transfection, the cells were seeded at  $3 \times 10^5$  cells per well onto coverslips in six-well plates and incubated overnight to obtain a subconfluent culture. The liposome-based reagent Tfx-20



**Fig. 1.** Progesterone increases the levels of E2- and E7-induced apoptosis. (a) HeLa cells were transiently transfected with the GFP-expressing plasmid pCMX-GFP3 and increasing amounts of either pWEB-E2, pWEB-E7 or the empty pWEB vector. After 24 h the percentage of apoptotic cells was determined as described in the text. The data are presented as the mean and standard deviation of four separate experiments. When error bars cannot be seen they are smaller than the symbols. (b) HeLa cells were transiently transfected with pCMX-GFP3 and either 300 ng pWEB-E2 or 800 ng pWEB-E7. The transfected cells were incubated in media containing progesterone at the concentrations indicated for 24 h. The percentage of apoptotic cells was then determined as above. The data are presented exactly as in (a). (c) The experiment shown in (b) was repeated using HeLa cells that had been grown in medium containing 2 μM progesterone for 1 week prior to transfection. (d) HeLa cells were grown in media containing the concentrations of progesterone indicated. The number of viable cells is proportional to the absorbance at 540 nm as determined in the MTT assay (see Methods). The values shown represent the mean of three experiments (when error bars cannot be seen they are smaller than the symbols).

(Promega) was used at a 3:1 liposome:DNA ratio in 1 ml serum-free medium per transfection, according to the manufacturer's instructions. After 18 h (E7 experiments) or 30 h (E2 experiments), the coverslips were washed in PBS and the cells fixed in 4% paraformaldehyde-PBS at 22 °C for 30 min. Following further washes in PBS, the cells were stained with bisbenzimidazole (Hoechst no. 33258; Sigma) for 30 min. Finally, the coverslips were washed in PBS and mounted onto microscope slides in 10 μl Mowiol (Calbiochem).

■ **Fluorescence microscopy.** Fluorescence microscopy was carried out using a Leica DM IRBE inverted epifluorescent microscope with FITC and DAPI filter sets and a 20 × air objective (Leica).

■ **MTT assays.** After removal of medium, cells were incubated in 5 μg/ml MTT [3-(4,5 dimethylthiazol 2-yl)-2,5-diphenyltetrazolium

bromide] for 2 h at 37 °C. The blue formazan crystals were then extracted and dissolved in DMSO, and the absorbance was measured at 560 nm with an ELISA plate reader (Mosmann, 1983).

## Results

E2- and E7-induced cell death shows all of the characteristic features of apoptosis including: chromatin condensation, blebbing of the plasma membrane and the appearance of cell fragments with sub-G<sub>0</sub> DNA content (Sanchez-Perez *et al.*, 1997; Desaintes *et al.*, 1997; Webster *et al.*, 2000). The plasmids pWEB-E2 and pWEB-E7 express the HPV-16 E2 and

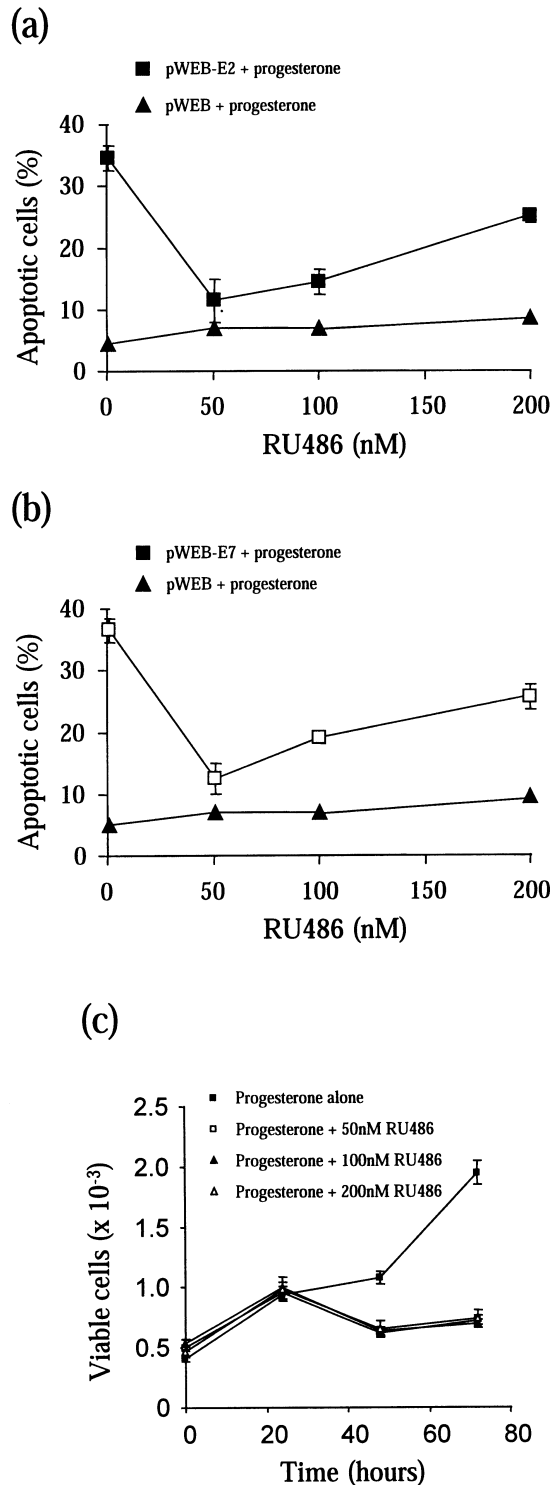


Fig. 2. RU486 blocks the effects of progesterone. (a) HeLa cells that had been grown in medium containing 2  $\mu$ M progesterone for 1 week were transiently transfected with pCMX-GFP3 and pWEB-E2. The transfected cells were incubated for 24 h in media containing 2  $\mu$ M progesterone and the concentrations of RU486 indicated. The percentage of apoptotic cells was then determined as in Fig. 1. (b) The experiment described in (a) was repeated using pWEB-E7. (c) HeLa cells that had been grown in medium containing 2  $\mu$ M progesterone for 1 week were placed in media

E7 proteins respectively under the control of the CMV promoter (Webster *et al.*, 2000). Increasing amounts of these plasmids or the empty pWEB vector were transiently cotransfected into HeLa cells (an HPV-18-transformed cervical carcinoma cell line) along with a plasmid that expresses GFP. After 24 h the cells were fixed and then stained with bisbenzimidazole (Hoechst stain). Individual cells were scored as untransfected or transfected on the basis of GFP expression and then examined for chromatin condensation and membrane blebbing using Hoechst stain and GFP, respectively (Webster *et al.*, 2000). At least 100 transfected cells were counted on each coverslip and the experiment was repeated four times. As can be seen from the data shown in Fig. 1(a), the presence of either pWEB-E2 or pWEB-E7 results in an increase in the percentage of cells undergoing apoptosis compared to cells transfected with the empty pWEB vector. Maximum levels of apoptosis are seen with 300 ng of pWEB-E2 and 800 ng of pWEB-E7.

To investigate any effects of steroid hormones on the induction of apoptosis by E2 and E7, we repeated the assay described above in the presence of increasing concentrations of progesterone. HeLa cells were transfected with pWEB-E2 or pWEB-E7 as before and then incubated in media containing different concentrations of progesterone. Under these conditions, progesterone has no effect on the induction of apoptosis by either protein (Fig. 1b). Although progesterone has little or no effect in this assay, prolonged exposure to progesterone can bring about cellular changes that are not seen after short-term treatment (for example see Groshong *et al.*, 1997). To determine whether prolonged exposure to progesterone might alter E2- and/or E7-induced apoptosis, we next cultured the cells in 2  $\mu$ M progesterone for 1 week prior to transfection. The results of this experiment are shown in Fig. 1(c). Interestingly, in this case there is a significant increase in the levels of E2- and E7-induced apoptosis in the presence of progesterone. In both E2- and E7-transfected cells levels of apoptosis increase from around 20% in the absence of progesterone to around 30% in the presence of 2  $\mu$ M progesterone. In both cases the levels of apoptosis seen in cells transfected with the empty pWEB vector remain at around 7%. Thus progesterone at these concentrations does not increase the background level of apoptosis in HeLa cells. To determine whether progesterone might have an effect on cell proliferation, we cultured HeLa cells in media containing increasing concentrations of progesterone and at 24 h intervals we determined the number of viable cells using MTT assays (Mosmann, 1983). Somewhat surprisingly, the presence of 2  $\mu$ M progesterone results in a decrease in cell proliferation (Fig. 1d). To determine whether progesterone might increase the levels of E2- and E7-induced apoptosis simply by increasing

containing the concentrations of progesterone and RU486 indicated. At the time-points indicated MTT assays were performed exactly as described in Fig. 1.

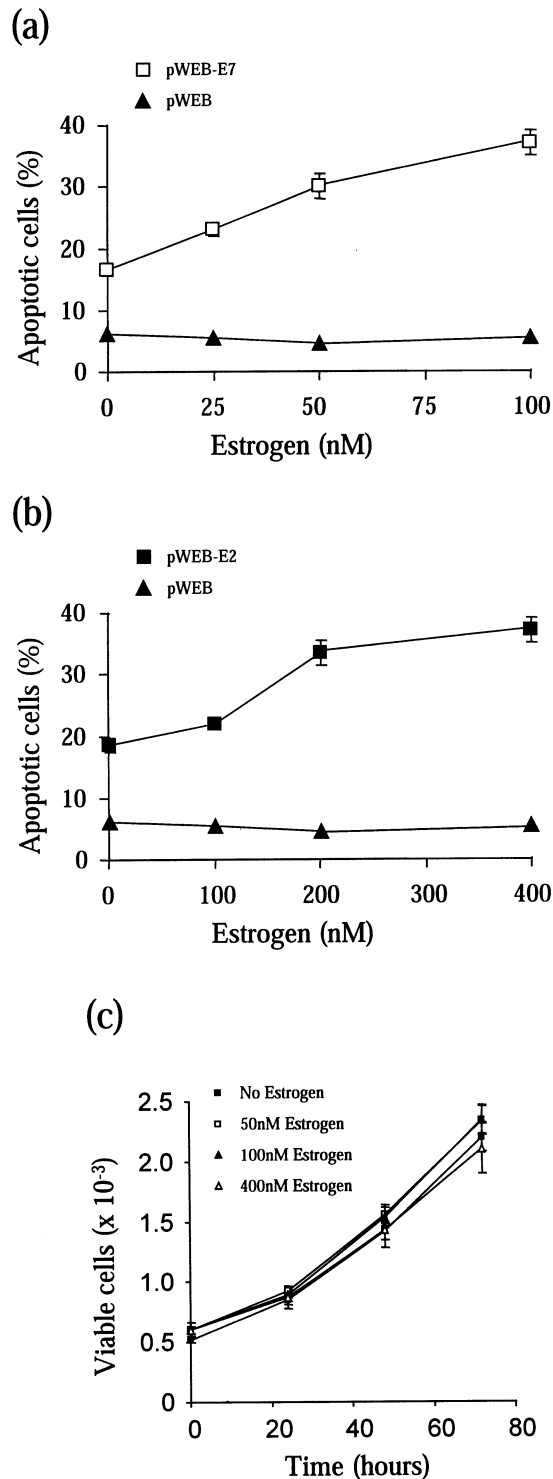


Fig. 3. Oestrogen increases the levels of E2- and E7-induced apoptosis. HeLa cells were transiently transfected with pCMX-GFP3 and pWEB-E7 and then incubated for 24 h in media containing the concentrations of oestrogen indicated. The percentage of apoptotic cells was determined as in Fig. 1. (b) The experiment described in (a) was repeated using pWEB-E2. (c) HeLa cells were incubated in media containing the concentrations of oestrogen indicated and MTT assays performed exactly as described in Fig. 1.

expression of the E2 and E7 genes carried on pWEB, we placed the luciferase gene downstream of the CMV promoter in pWEB and assayed luciferase activity after transient transfection. The addition of progesterone had no effect on luciferase activity suggesting that under these conditions the CMV promoter does not respond to this hormone (data not shown).

### Progesterone increases E2- and E7-induced apoptosis via the progesterone receptor

Progesterone binds to the progesterone receptor and induces a conformational change in the receptor that allows the hormone–receptor complex to bind to specific DNA sequences and regulate gene expression. The anti-progesterone compound RU486 (Mifepristone) binds with high affinity to the progesterone receptor and acts as a partial antagonist (reviewed by Cadepond *et al.*, 1997). To determine whether the progesterone receptor is required for the increase in E2- and E7-induced cell death seen in the presence of progesterone, we cultured HeLa cells in 2  $\mu$ M progesterone for 1 week prior to transfection with the plasmids pWEB-E2 and pWEB-E7. The transfected cells were then incubated in media containing 2  $\mu$ M progesterone and increasing concentrations of RU486. As expected, the cell populations transfected with either the E2 or the E7 expression plasmid and incubated in 2  $\mu$ M progesterone show high levels of apoptosis (Fig. 2a, b, respectively). In contrast, in the presence of progesterone and 50 nM RU486 the levels of E2- and E7-induced apoptosis are reduced almost to the background levels seen in the population transfected with the empty vector. RU486 can also bind to the glucocorticosteroid receptor and act as an anti-glucocorticosteroid. However, the glucocorticosteroid dexamethosone has little if any effect on the levels of E2- or E7-induced apoptosis, suggesting that in this case the effects of RU486 are not due to its binding to the glucocorticosteroid receptor (not shown). Taken together, these experiments suggest that the progesterone receptor is required for progesterone to elicit its effect on E2- and E7-induced apoptosis.

Interestingly, at higher concentrations of RU486 (100 and 200 nM), the levels of E2- and E7-induced apoptosis are raised compared to those seen at 50 nM RU486. However, in the presence of progesterone and high concentrations of RU486 there is also a slight increase in the level of apoptosis seen in the cells transfected with the empty pWEB vector. To examine the effects of progesterone and RU486 on cell proliferation we performed MTT assays exactly as described above. Interestingly, continuous culture in the presence of progesterone and RU486 has a much greater negative effect on cell proliferation than progesterone alone (Fig. 2c). Thus, the increased levels of cell death seen in the presence of progesterone and high concentrations of RU486 are probably due to toxicity of this combination of hormones rather than an effect of these hormones on E2- and E7-induced apoptosis.

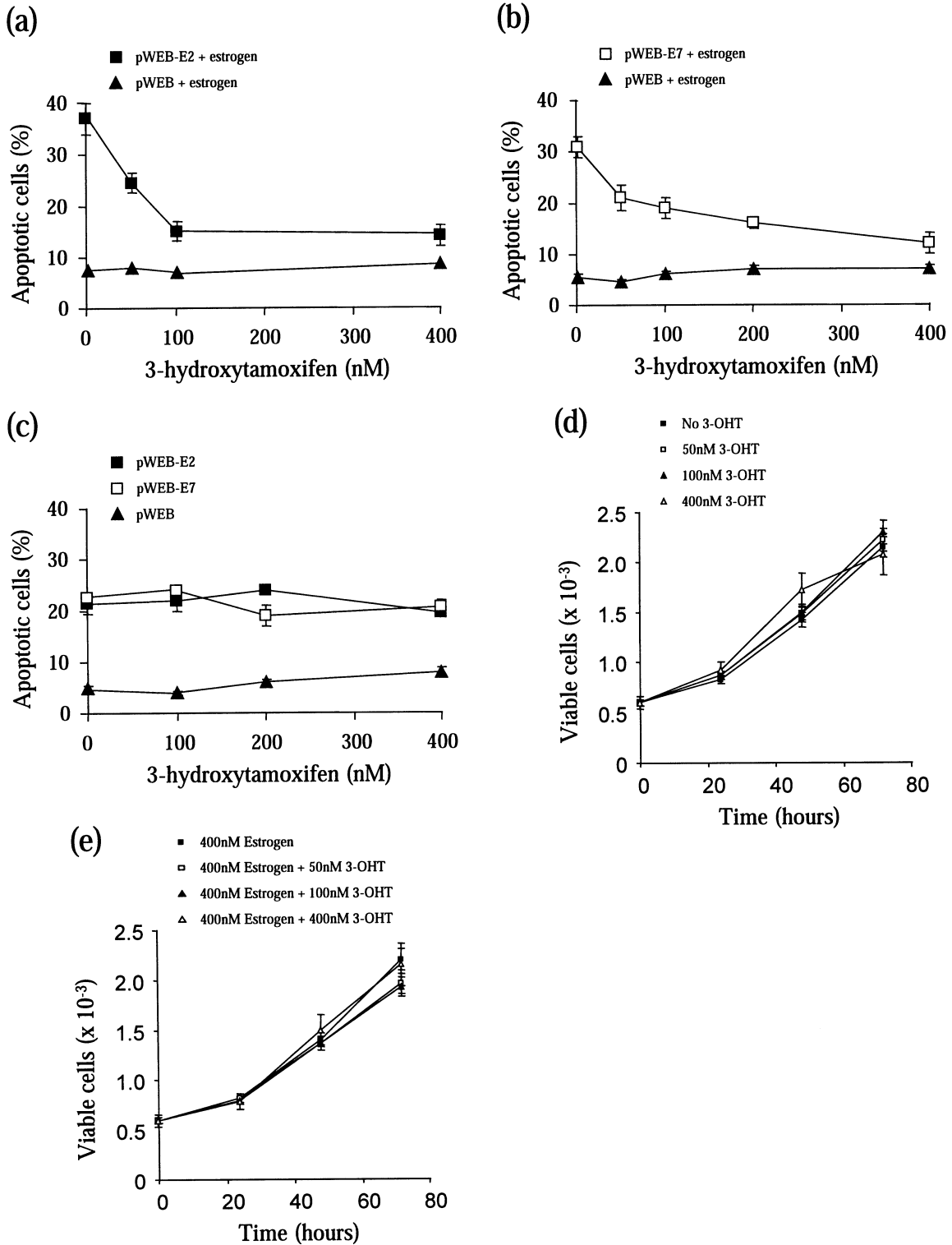


Fig. 4. 3-Hydroxytamoxifen blocks the effects of oestrogen. (a) HeLa cells were transiently transfected with pCMX-GFP3 and pWEB-E2 and then incubated for 24 h in media containing 400 nM oestrogen and the concentrations of 3-hydroxytamoxifen indicated. The percentage of apoptotic cells was then determined as in Fig. 1. (b) The experiment described in (a) was repeated using pWEB-E7 and 100 nM oestrogen. (c) HeLa cells were transiently transfected with pCMX-GFP3 and either 300 ng pWEB-E2 or 800 ng pWEB-E7. The transfected cells were incubated in media containing 3-hydroxytamoxifen at the concentrations indicated for 24 h. The percentage of apoptotic cells was then determined as above. (d) HeLa cells were grown in media containing the concentrations of 3-hydroxytamoxifen indicated and MTT assays were performed exactly as described

### Oestrogen increases the levels of E2- and E7-induced apoptosis

To investigate any effects of oestrogen on E2- and E7-induced apoptosis we transiently transfected HeLa cells with pWEB-E2, pWEB-E7 or the empty pWEB vector and then incubated the cells in media containing different concentrations of 17- $\beta$ -oestradiol, the primary active oestrogen. As expected, transfection with the E7-expressing plasmid results in a significant increase in the level of apoptosis when compared to cells transfected with the empty vector (Fig. 3*a*). In the presence of oestrogen there is a significant increase in the level of E7-induced apoptosis. The percentage of E7-expressing cells undergoing apoptosis increases from around 20% in the absence of oestrogen to around 40% in the presence of 100 nM oestrogen. In contrast, the level of apoptosis in the cells transfected with the empty pWEB vector remains at around 5–7% in both the presence and absence of oestrogen. Oestrogen also brings about a significant increase in the level of E2-induced apoptosis (Fig. 3*b*). However, in this case higher concentrations of oestrogen (between 200 and 400 nM) are required to bring about an increase from around 20% apoptotic cells in the absence of oestrogen to around 40% in the presence of oestrogen. These higher oestrogen concentrations do not increase the level of apoptosis in cells transfected with the empty vector. Thus, oestrogen increases the levels of E7- and E2-induced apoptosis but does not increase the background level of apoptosis in these cells.

To determine whether the concentrations of oestrogen used in these experiments have any effects on cell proliferation we performed MTT assays. Continuous culture for 72 h in the presence of up to 400 nM oestrogen has no effect on the proliferation of these cells (Fig. 3*c*). This, and the absence of increased apoptosis in the pWEB-transfected population, suggests that the increases in the levels of inducible apoptosis are a specific response, and not due to a general toxicity of the hormone. As in the case of progesterone, oestrogen had little or no effect on the activity of a CMV-luciferase construct, suggesting that under these conditions the CMV promoter is not responsive to oestrogen (data not shown).

### Oestrogen increases E2- and E7-induced apoptosis via an oestrogen receptor

Oestrogen binds to oestrogen receptors resulting in a conformational change that allows the hormone-receptor complexes to regulate the expression of target genes. The oestrogen receptor antagonist 3-hydroxytamoxifen (3-OHT) binds to oestrogen receptors and blocks receptor activity. To determine whether oestrogen receptors are required for the

increase in E2- and E7-induced cell death seen in the presence of oestrogen, we transfected E2 and E7 expression plasmids into HeLa cells and then incubated the cells in media containing oestrogen and increasing concentrations of 3-OHT. As expected, cells transfected with pWEB-E2 or pWEB-E7 and incubated in the presence of 400 and 100 nM oestrogen respectively, show high levels of apoptosis (Fig. 4*a, b*). However, the percentage of apoptotic cells is reduced from around 35% in the presence of E2 and oestrogen to around 13% in the presence of E2, oestrogen and 400 nM 3-OHT (Fig. 4*a*). Similarly, the percentage of apoptotic cells is reduced from around 35% in the presence of E7 and oestrogen to around 15% in the presence of E7, oestrogen and 400 nM 3-OHT (Fig. 4*b*). In each experiment the percentage of apoptotic cells in the pWEB-transfected population is not altered in the presence of oestrogen and 3-OHT. In the absence of oestrogen, 3-OHT has little or no effect on the levels of E2- or E7-induced apoptosis (Fig. 4*c*). Similarly, MTT assays show that 3-OHT and the combination of 3-OHT and oestrogen have little or no effect on the proliferation of these cells (Fig. 4*d, e*, respectively). Taken together these data suggest that the effects of oestrogen on the levels of E2- and E7-induced apoptosis are mediated by an oestrogen receptor.

### 16 $\alpha$ -Hydroxylation is probably required for the effects of oestrogen on E2- and E7-induced apoptosis

Oxidation of 17- $\beta$ -oestradiol to oesterone can be followed by hydroxylation to give 2-hydroxyoesterone or 16 $\alpha$ -hydroxyoestrone. Whilst 2-hydroxyoesterone is a weak anti-oestrogen, 16 $\alpha$ -hydroxyoestrone is highly oestrogenic and might be tumorigenic. Indole-3-carbinol (I3C) induces 2 $\alpha$ -hydroxylation of oesterone and thereby reduces 16 $\alpha$ -hydroxylation (Michnovicz & Bradlow, 1990). To determine whether I3C can influence the levels of apoptosis induced by E2 and oestrogen and E7 and oestrogen, we repeated the transfections described above and incubated the cells in media containing oestrogen and either 50 or 100  $\mu$ M I3C. As can be seen from the data, I3C reduces the levels of apoptosis induced by E2 and oestrogen (Fig. 5*a*) and E7 and oestrogen (Fig. 5*b*). These data suggest that altering the 2 $\alpha$ -hydroxylation/16 $\alpha$ -hydroxylation ratio in favour of the 2 $\alpha$ -hydroxyoestrone metabolite can significantly reduce the effects of oestrogen on E2- and E7-induced cell death. In the absence of supplemental oestrogen, I3C brings about a minor reduction in the levels of E2- and E7-induced apoptosis (Fig. 5*c*) and MTT assays show that I3C alone, and I3C together with oestrogen, bring about a slight reduction in cell proliferation (Fig. 5*d, e*). This raises the possibility that I3C might reduce cell death by a mechanism that does not involve changes in oestrogen metabolism.

in Fig. 1. (e) HeLa cells were grown in media containing the concentrations of oestrogen and 3-hydroxytamoxifen indicated and MTT assays were performed exactly as above.

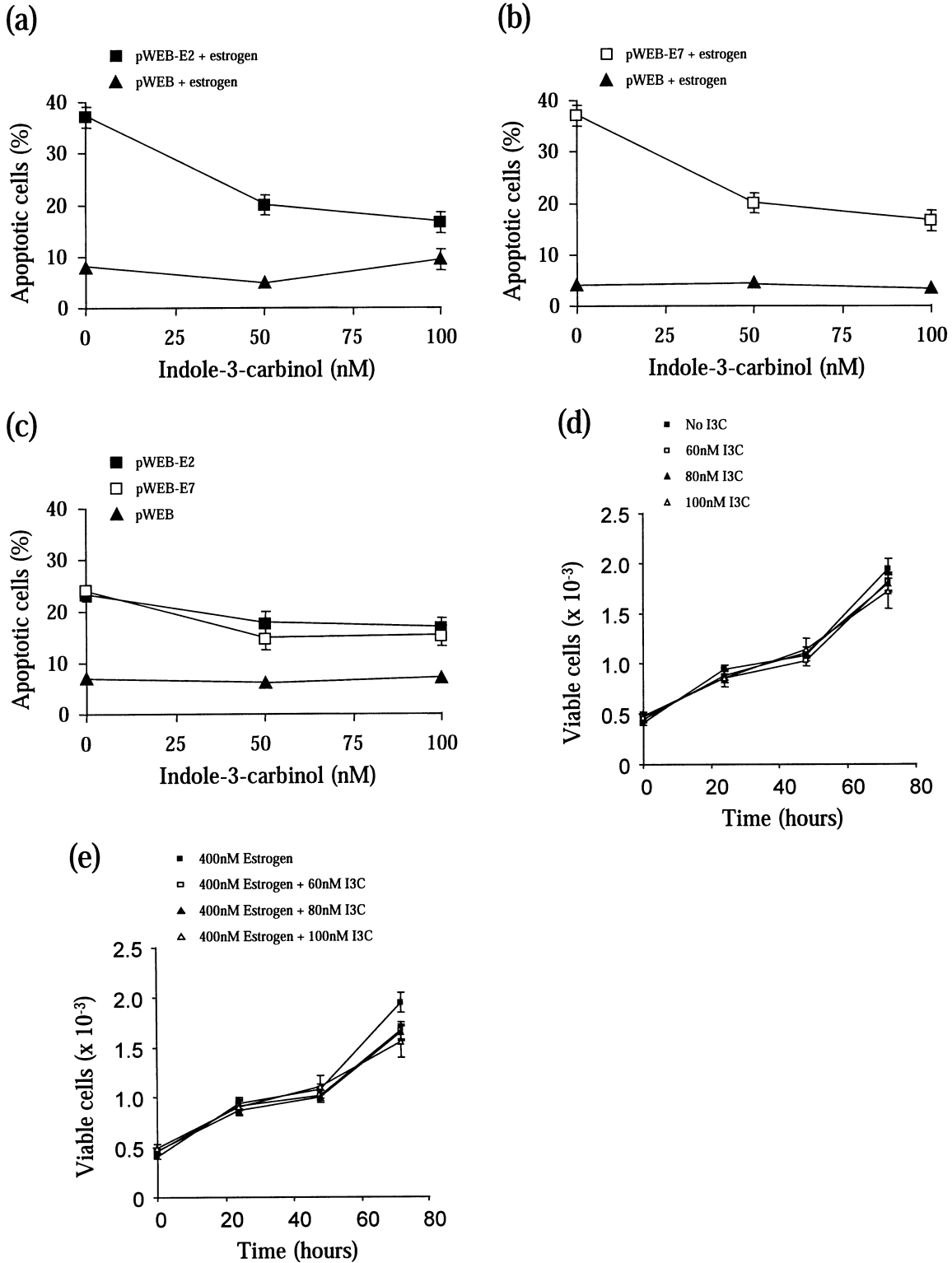


Fig. 5. Indole-3-carbinol blocks the effects of oestrogen. HeLa cells were transiently transfected with pCMX-GFP3 and pWEB-E2 and then incubated for 24 h in media containing 400 nM oestrogen and the concentrations of indole-3-carbinol indicated. The percentage of apoptotic cells was then determined as in Fig. 1. (b) The experiment described in (a) was repeated using pWEB-E7 and 100 nM oestrogen. (c) HeLa cells were transiently transfected with pCMX-GFP3 and either 300 ng pWEB-E2 or 800 ng pWEB-E7. The transfected cells were incubated in media containing indole-3-carbinol at the concentrations indicated for 24 h. The percentage of apoptotic cells was then determined as above. (d) HeLa cells were grown in media containing the

However, these effects could also be due to I3C blocking the effects of oestrogenic compounds present in the culture media.

To confirm and extend these results we next looked at whether  $16\alpha$ -hydroxyoestrone might mimic the effects of oestrogen on E2- and E7-induced cell death. HeLa cells were transiently transfected with E2- and E7-expressing plasmids and then incubated in media containing increasing amounts of  $16\alpha$ -hydroxyoestrone. The addition of  $16\alpha$ -hydroxyoestrone increases the levels of both E2- and E7- induced apoptosis (Fig. 6a, b, respectively). However, I3C is unable to bring about a decrease in the levels of apoptosis induced by E2 and  $16\alpha$ -hydroxyoestrone or E7 and  $16\alpha$ -hydroxyoestrone (Fig. 6c). Taken together these data suggest that the effects of oestrogen on E2- and E7-induced cell death are mediated by  $16\alpha$ -hydroxyoestrone. Since the presence of I3C favours  $2\alpha$ -hydroxylation of oestrogen over  $16\alpha$ -hydroxylation, I3C can block the effects of oestrogen but cannot block the effects of  $16\alpha$ -hydroxyoestrone.

### RU486 blocks the effects of oestrogen on E2- and E7-induced apoptosis

To investigate any possible role for the progesterone receptor in the enhancement of E2- and E7-induced cell death by oestrogen, we transfected HeLa cells with either the E2 or the E7 expression plasmid and then incubated the cells in media containing oestrogen and increasing amounts of RU486. The populations of cells transfected with the E2 or E7 expression plasmid and incubated in media containing oestrogen alone show high levels of apoptosis (Fig. 7a, b, respectively). In contrast, cells transfected with these plasmids and then incubated in media containing oestrogen and RU486 show levels of apoptosis that are only slightly above background. MTT assays show that RU486 alone and the combination of oestrogen and RU486 have little or no effect on cell proliferation (Fig. 7c, d, respectively). In contrast, the combination of RU486 and progesterone brings about a marked reduction in cell proliferation (Fig. 2c). Taken together these experiments suggest that the progesterone receptor might also be required for oestrogen to elicit its effect on E2- and E7-induced apoptosis.

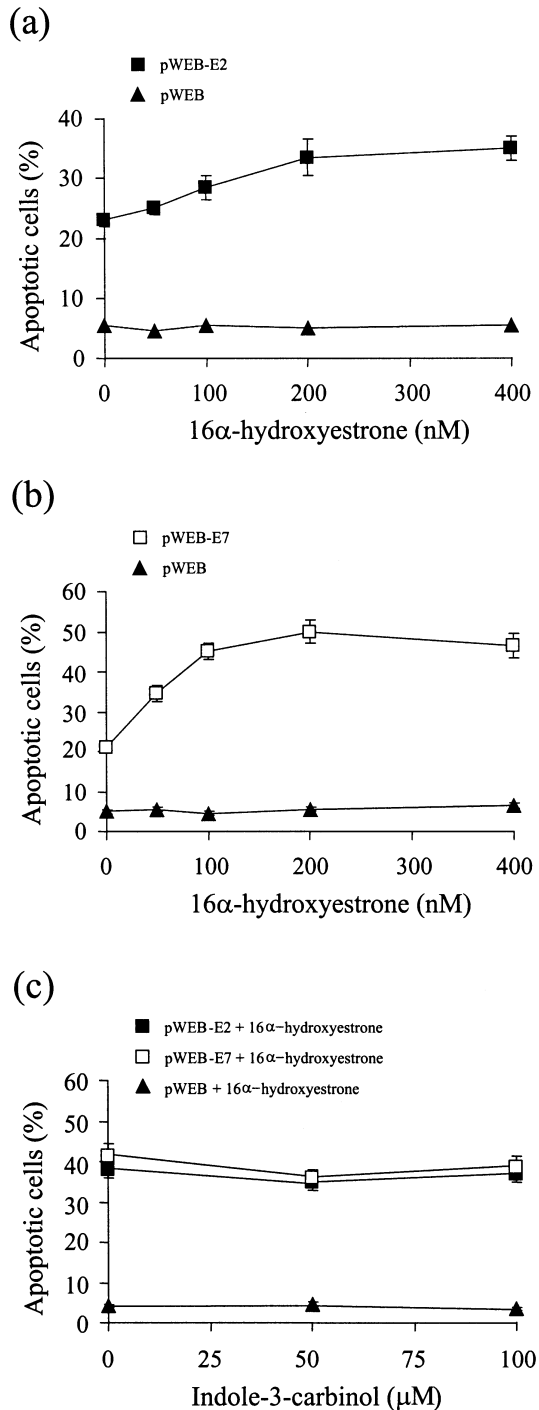
## Discussion

In conjunction with certain high-risk human papillomavirus types, such as HPV-16, progesterone and oestrogen are thought to be cofactors in cervical carcinogenesis. However, the exact mechanism by which these hormones increase cancer risk is not known. We and others have shown that the HPV-16

E2 and E7 proteins are both capable of inducing apoptosis in HPV-transformed and non-HPV-transformed cells (Sanchez-Perez *et al.*, 1997; Desaintes *et al.*, 1997; Frattini *et al.*, 1997; Webster *et al.*, 2000). Here we have shown that progesterone and oestrogen can increase the levels of E2- and E7-induced apoptosis in HeLa cells, an HPV-18-transformed cervical carcinoma cell line. Whilst the increase in E2- and E7-induced apoptosis brought about by oestrogen and progesterone would appear to be inconsistent with the hypothesis that these hormones might be cofactors in HPV-induced tumorigenesis, it is important to point out that both E2- and E7-induced apoptosis can be blocked by the HPV E6 protein via inactivation of p53 (Webster *et al.*, 2000). Presumably, in a virus-infected cell the pro-apoptotic signals from E2 and E7 are counter-balanced by an anti-apoptotic signal from E6. Although steroid hormones can increase E2- and E7-induced apoptosis and thereby affect this balance, this might not be sufficient to result in a decrease in cell proliferation and a consequent decrease in cancer risk. Furthermore, the full effects of these hormones *in vivo* are still not completely understood. For example, experiments with transgenic mice in which expression of the HPV-16 E6 and E7 genes is under the control of the keratin-14 promoter have suggested that there is a synergism between oestrogen and these oncogenes that results in increased cancer risk independently of changes in HPV gene expression (Arbeit *et al.*, 1996). One possible explanation for this synergism is that long-term exposure to oestrogen causes DNA damage that could act with E7 to bring about tumorigenesis (Auborn *et al.*, 1991; Telang *et al.*, 1992).

Several previous studies have shown that HPV gene expression can be increased in response to either progesterone and oestrogen (Mitrani-Rosenbaum *et al.*, 1989; Mittal *et al.*, 1993; Chen *et al.*, 1996; Yuan *et al.*, 1999a; Kim *et al.*, 2000). Thus one way in which these hormones might bring about an increase in the levels of E2- and E7-induced cell death in HeLa cells is via a direct effect upon expression of the integrated HPV-18 E6 and/or E7 genes. At the concentrations used in our experiments neither progesterone nor oestrogen has any effect on the levels of apoptosis seen in cells that are not expressing E2 or exogenous E7. These data suggest that the ability of these hormones to augment the levels of E2- and E7-induced cell death may not simply be a consequence of their effects on expression of the integrated E6 and E7 genes. However, whether progesterone and oestrogen increase E2- and E7-induced cell death via direct effects on HPV gene expression, or via indirect pathways, our experiments demonstrate that these hormones are important in determining the outcome of E2 and E7 expression.

concentrations of indole-3-carbinol indicated and MTT assays were performed exactly as described in Fig. 1. (e) HeLa cells were grown in media containing the concentrations of oestrogen and indole-3-carbinol indicated and MTT assays were performed exactly as above.



**Fig. 6.** 16 $\alpha$ -Hydroxyoestrone increases the levels of E2- and E7-induced apoptosis. HeLa cells were transiently transfected with pCMX-GFP3 and pWEB-E2 and then incubated for 24 h in media containing the concentrations of 16 $\alpha$ -hydroxyoestrone indicated. The percentage of apoptotic cells was then determined as in Fig. 1. (b) The experiment described in (a) was repeated using pWEB-E7. (c) HeLa cells were transiently transfected with pCMX-GFP3 and either 300 ng pWEB-E2 or 800 ng pWEB-E7. The transfected cells were incubated in media containing 16 $\alpha$ -hydroxyoestrone and indole-3-carbinol at the concentrations indicated for 24 h. The percentage of apoptotic cells was then determined as above.

The oestrogen receptor antagonist 3-hydroxytamoxifen blocks the increases in E2- and E7-induced cell death seen in the presence of oestrogen, but has no effect on the levels of E2- or E7-induced cell death seen in the absence of oestrogen, or on the background levels of cell death. Similarly, the anti-progesterone RU486 (Mifepristone) blocks the increases in E2- and E7-induced cell death seen in the presence of progesterone. Somewhat surprisingly, RU486 can also block the increases in E2- and E7-induced cell death seen in the presence of oestrogen. These data suggest that oestrogen acts upstream of progesterone (or at least the progesterone receptor) in a pathway that leads to increased cell death in the presence of over-expressed E2 or E7. One possibility is that oestrogen increases the levels of progesterone receptor within HeLa cells and that in the presence of progestins this leads to increased E2- and E7-induced cell death. Another possibility is that RU486 blocks E2- and E7-induced cell death indirectly. Both E2 and E7 induce p53-dependent apoptosis in at least some experimental systems (Webster *et al.*, 2000) and RU486 has been shown to decrease the levels of p53 in breast cancer cells (Hurd *et al.*, 1995). However, RU486 has been shown to have little or no effect on p53 levels in C4-1 cervical carcinoma cells (Kamradt *et al.*, 1999).

Oestrogen metabolism produces a family of related compounds including the highly oestrogenic 16 $\alpha$ -hydroxyoestrone and the weakly anti-oestrogenic 2-hydroxyoestrone. Cells in the transformation zone of the cervix exhibit a constitutively high level of conversion of oestrogen to 16 $\alpha$ -hydroxyoestrone (Auborn *et al.*, 1991). We have shown that 16 $\alpha$ -hydroxyoestrone can also increase the levels of both E2- and E7-induced apoptosis. Indole-3-carbinol is a dietary compound that induces 2-hydroxylation of oestrogen at the expense of 16 $\alpha$ -hydroxylation and that has been proposed as a potential preventative of cervical cancer (Michnovicz & Bradlow, 1990; Yuan *et al.*, 1999b). We have shown that indole-3-carbinol blocks the stimulatory effect of oestrogen on the levels of E2- and E7-induced cell death but does not block the stimulatory effect of 16 $\alpha$ -hydroxyoestrone. Therefore, the 16 $\alpha$ -hydroxylation of oestrogen is probably required for the effect of this hormone on E2- and E7-induced cell death. However, indole-3-carbinol has been shown to induce cell cycle arrest in breast cancer cells through the inhibition of cyclin-dependent kinase-6 gene expression (Cover *et al.*, 1998). Thus it is also possible that it might block the increase in E2- and E7-induced apoptosis seen in the presence of oestrogen via a mechanism that does not involve changes in oestrogen metabolism.

In HPV-induced cervical cancer, HPV DNA is often integrated into the host genome and this frequently results in the loss of the E2 protein. Since the E2 protein can modulate HPV gene expression, virus integration results in deregulated expression of E6 and E7. Furthermore, since HPV E2 protein is a potent inducer of apoptosis, the loss of E2 might be expected to result in increased cell proliferation (Sanchez-Perez *et al.*, 1997; Webster *et al.*, 2000). We have shown here that

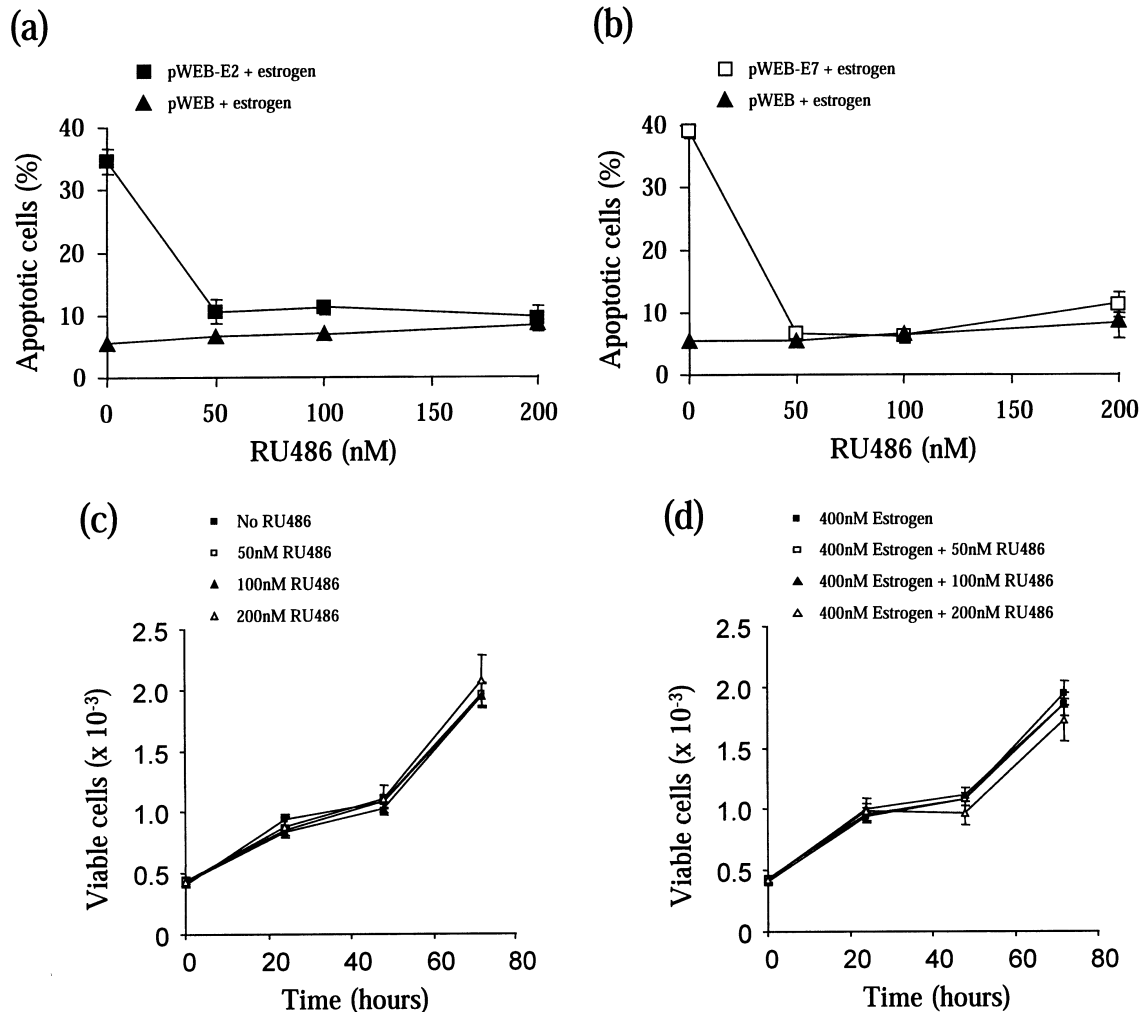


Fig. 7. RU486 blocks the effects of oestrogen. (a) HeLa cells were transiently transfected with pCMX-GFP3 and pWEB-E2. The transfected cells were incubated for 24 h in media containing 400 nM oestrogen and the concentrations of RU486 indicated. The percentage of apoptotic cells was determined as in Fig. 1. (b) The experiment described in (a) was repeated using pWEB-E7. (c) HeLa cells were placed in media containing the concentrations of RU486 indicated. At the time-points indicated MTT assays were performed exactly as described in Fig. 1. (d) HeLa cells were grown in media containing the concentrations of oestrogen and RU486 indicated and MTT assays were performed exactly as above.

oestrogen and progesterone increase the levels of E2-induced cell death. One possibility is that in the presence of E2 these hormones might be protective against cervical cancer via their upregulation of cell death. In contrast, in the absence of E2 these hormones might be a risk factor in cervical carcinogenesis either via their effects on HPV or cellular gene expression, or via other, as yet ill-defined, pathways. Agents such as indole-3-carbinol and 3-hydroxytamoxifen that block the effects of oestrogens, and RU486 that blocks the effects of progestins, could also have different effects on cancer risk before and after HPV integration. Our results highlight the need for further work in this area.

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