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c-*ras*^{H} and ornithine decarboxylase are induced by oestradiol-17β in the mouse uterine luminal epithelium independently of the proliferative status of the cell

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Oestradiol-17β (E_2) treatment of the ovariectomized mouse results in a synchronised wave of cell proliferation in the uterine luminal epithelium. At the peak of DNA synthesis the mRNA level of the c-*ras*^{H} proto-oncogene and ornithine decarboxylase were significantly increased. Progesterone treatment completely inhibits the E_2 induced wave of DNA synthesis but does not greatly influence the level of these 2 mRNAs. Thus in the uterine luminal epithelium E_2 regulates the level of ornithine decarboxylase and c-*ras*^{H} independently of cell proliferation.

Estradiol-17β *Progesterone* *Ornithine decarboxylase* *Proto-oncogene*

1. INTRODUCTION

Over the years many proteins have been implicated as positive regulators of cell proliferation. Ornithine decarboxylase (ODC) the rate limiting enzyme of polyamine synthesis [1–3], was thought to play such a role [4,5] because of its short half-life and its rapid inducibility by proliferation signals [1,2]. Kinetic considerations, however, have shown that this enzyme is unlikely to be regulatory for the initiation of DNA synthesis although it appears to be an integral part of the growth pathway [6,7]. Now it is proposed that the proto-oncogenes play positive regulatory functions in cell proliferation because of their association with transforming retroviruses [8]. Indeed, one family of these proteins, the *ras* family, stimulates DNA synthesis when injected into fibroblasts in culture [9,10]. Because of this effect on DNA synthesis and the association of *ras* with carcinomas [11,12] it is important to study the role of *ras* in epithelial tissues in vivo. A convenient system to do this is to use the luminal epithelium of the mouse uterus following treatment with female sex steroids. This is because oestradiol-17β (E_2) given alone to ovariectomized adult mice induces a synchronised wave of DNA synthesis and cell division in this epithelium [13,14]. Four daily injections of progesterone however, completely inhibits this wave of cell proliferation [15] but has no effect on the time course or extent of the E_2 stimulation of protein or rRNA synthesis [16,17]. Furthermore, since the luminal epithelium can be isolated with great purity from the underlying stroma [18] biochemical analysis may be performed on this tissue. Using this system we have shown that E_2 induces the expression of c-*ras*^{H} proto-oncogene and ODC independently of cell proliferation.

2. MATERIALS AND METHODS

Ovariectomized adult Schneider mice were primed with 2 injections of 100 ng E_2 1 week before being killed and at least 2 weeks after

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ovariectomy. After priming the mice were treated with one of the following hormone treatments [17,19] given sub-cutaneously in arachis oil. (i) Control animals given no hormone treatment. (ii) Animals given a single 50 ng injection of E_2 6 days after priming, a treatment that results in a synchronized wave of epithelial cell proliferation [14]. (iii) Animals given 4 daily injections of 1 mg progesterone commencing 2 days after priming. (iv) Animals given progesterone as in (iii) with 50 ng E_2 on the fourth day. This progesterone treatment completely suppresses the oestradiol induced wave of epithelial cell proliferation [15]. At appropriate time thereafter, animals were killed and uterine luminal epithelial homogenates free from the remainder of the uterus prepared by the method of Fagg et al. [18]. From this homogenate total RNA was isolated by the method of Chirgwin et al. [20]. 20 or 50 μg of total RNA, following formamide denaturation, was subjected to formaldehyde, 1% agarose gel electrophoresis [21] using restriction digested lambda DNA, *E. coli* and *Chorella* ribosomal RNA as M_r markers. Following electrophoresis the RNA was transferred to nitrocellulose and probed with either pBS-9 insert (*ras*^{H}) or pOD48 (ODC) nick translated with [^{32}P]dCTP. Plasmids were isolated by the method of Birnboim and Doly [23] and further purified by ethidium bromide-caesium chloride centrifugation. The plasmid pBS-9 [22] contained a 0.45 kb insert of the v-*ras*^{H} oncogene inserted in the *Eco*RI site of pBR322. This insert was purified from low melting point agarose gels after *Eco*RI digestion. pOD48 [24] contained a 1.6 kb cDNA insert homologous to most of the coding region of ODC and the whole plasmid was used in the hybridization reaction. Northern blots were probed with either of the above cloned sequences for 60 h at 42°C using the conditions described by Taylor et al. [25]. The blots were washed to a final stringency of 0.1 × SSC at 52°C, dried and subjected to autoradiography using Kodak X-Omat film with intensifying screens at −70°C. Resultant autoradiograms were scanned using a Zeineh soft scanning laser densitometer and the area under the scan determined from triplicate scans after the subtraction of a background derived from an empty lane of the Northern blot.

To estimate ODC enzyme activity luminal epithelial homogenates were prepared from 10 mice in the control group and 5 mice in the various treatment groups and ODC activity estimated by the method of Djurhuus [26]. Protein content was measured by the method of Lowry et al. [27]. The ODC assay was linear with protein content and over the time course of the assay. It was shown to be specific for ODC by the complete inhibition of activity with the irreversible competitive inhibitor of ODC, α-difluoromethylornithine [28].

3. RESULTS

To assess the effect of E_2 on c-*ras*^{H} and ODC mRNA total uterine epithelial RNA was isolated 6 and 12 h after E_2 injection and subjected to Northern blot analysis and the level of each of these mRNAs compared to the level in the untreated control. E_2 increased the relative cytoplasmic content of a single 1.4 kb mRNA species homologous to v-*ras*^{H} gene in the proliferative epithelium. There was a significant increase in mRNA at 6 h and a 9-fold stimulation by 12 h (fig.1, table 1).

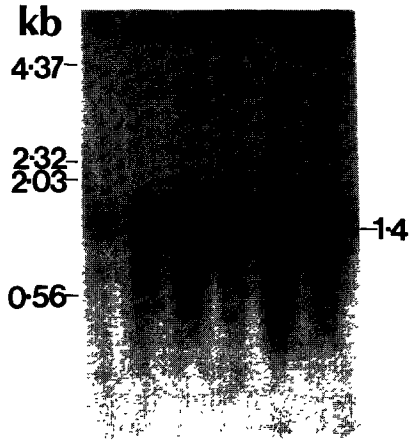

Fig.1. Analysis of the expression of c-*ras*^{H} in relationship to cellular proliferation in the mouse uterine luminal epithelium. Autoradiogram of a Northern blot of total uterine luminal epithelial RNA probed with a v-*ras*^{H} genomic clone. Each lane has 50 μg RNA isolated from the luminal epithelium of ovariectomized adult Schneider mice treated in the following ways (lanes): no treatment (1), a single 50 ng injection of E_2 6 h (2) or 12 h (3) before being killed, animals given 1 mg progesterone per day for 4 days (4), or given 50 ng E_2 after 4 previous daily treatments of progesterone and killed 6 (5) or 12 h (6) after injection.

Table 1

Densitometric quantitation of the levels of c-*ras*^{H} and ornithine decarboxylase mRNA from the Northern blot analysis

Hormone treatment	Hours after oestradiol-17β	Area under the scan (mm^2)					
		c-*ras*^{H} (1.4 kb)			ODC (2.2 kb)		
		(1)	(2)		(1)	(2)	
Oestradiol-17β alone	0	142	377	(−)	36	98	(−)
	6	432	1325	(3.3)	143	608	(5.1)
	12	1264	3163	(8.7)	665	2761	(23.3)
Progesterone: oestradiol-17β	0	435	1245	(3.2)	248	950	(8.3)
	6	745	1871	(5.1)	355	904	(9.5)
	12	1283	2799	(8.3)	628	1591	(16.8)

20 μg (1) or 50 μg (2) of total RNA isolated from mouse uterine epithelial cells was subjected to Northern blot analysis and autoradiograms quantitated using a Zeineh soft laser scanning densitometer. The average factorial increase from the 2 RNA samples shown (in parentheses) is the amount of stimulation compared to the luminal epithelial mRNA level in a relevant untreated ovariectomized control. Staining of parallel tracks with toludine blue showed that the amount of RNA in each track was indistinguishable

Progesterone, however, despite its complete inhibition of DNA synthesis [15] did not significantly effect the accumulation of this mRNA in response to E_2 (fig.1, table 1). In fact even in the absence of E_2 progesterone caused a reproducible elevation in the c-*ras*^{H} mRNA level (table 1). A similar pattern of response was observed with ODC mRNA. In this case the mRNA level was stimulated by E_2 5-fold at 6 h and 23-fold by the peak of DNA synthesis (fig.2 and table 1). Four days of progesterone treatment failed to prevent the increase in mRNA level and, in fact, caused an 8-fold increase even in the absence of E_2. The major mRNA species was a band of approx. 2.2 kb. Upon longer exposures a higher-M_r band at 2.7 kb was also detected at an elevated level following E_2 treatment. A band at 1.8 kb was also observed whose relative content was also increased by E_2 treatment but interestingly not following progesterone pretreatment. We also measured ODC enzyme activity [26] during the first 6 h of E_2 treatment. ODC activity underwent a dramatic stimulation within 4 h of E_2 treatment, reaching a 60-fold stimulation within 6 h. Progesterone pretreatment did not inhibit this E_2 induced increase in activity which under this regime showed a 75-fold increase within 6 h. Interestingly again, progesterone stimulated the enzyme activity to a small extent in the absence of E_2.

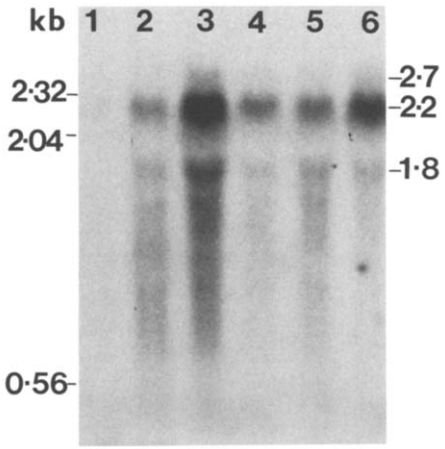

Fig.2. Analysis of the level of ornithine decarboxylase mRNA in the mouse uterine epithelium. 50 μg total RNA isolated from the epithelium of animals given the hormone regimes described in fig.1 was subjected to Northern gel analysis and probed with a ^{32}P nick translated pOD48 plasmid. Lanes 1–6 are comparable to those described in fig.1.

4. DISCUSSION

E_2 induces luminal epithelial DNA synthesis which begins 6–8 h after administration of the hormone and reaches a maximum at 12–15 h [13,14]. At this time essentially all the cells are engaged in

DNA synthesis. By 15 h the cells enter into mitosis and the cell number doubles [13]. Progesterone pretreatment, however, completely suppresses this proliferative response [15]. E_2 induced a major 2.2 kb ODC mRNA band and minor 2.7 and 1.8 kb bands in the luminal epithelium. The major and minor bands are similar to those observed in other mouse tissues [24,29,30] and may represent alternatively spliced mRNAs or transcripts from different ODC genes. The pattern of E_2 stimulation of ODC enzyme activity was comparable to that described in other tissues exposed to growth factors [31] and to that described in the whole immature rat uterus in response to E_2 [32]. Quantitatively however, the increased level of ODC mRNA seems unlikely to explain all the stimulation of the ODC enzyme activity (tables 1 and 2) suggesting that E_2 also affects the activity or stability of this enzyme. In other cell types treated with growth factors a modulation of both these paramaters resulting in increased ODC levels has been described [31].

This pattern of ODC stimulation which is closely correlated with an increase in cell growth and not with cell proliferation suggests that ODC is unlikely to be directly involved in the regulation of cell division as has been concluded by other workers [5,6]. It might however, be indirectly involved since Russell [33] has presented evidence that ODC is a stimulatory factor for RNA polymerase I. Increased rRNA synthesis is invariably seen in cells stimulated to divide and may explain why there is such a large stimulation of ODC activity over and above the apparent requirement for polyamines following mitogen treatment. The lack of correlation seen between ODC activation, rRNA synthesis and cell proliferation [17,34] in the luminal epithelium may be explained by the recent observations that cell growth and cell proliferation are independently but coordinately regulated pathways [17,35]. Thus the former 2 events would normally be associated with cell proliferation but can be regulated independently from it. It should be pointed out, however, that ODC activation lags behind the E_2 stimulation of rRNA synthesis. A dissociation of time course between cell growth and ODC activity has also been described in the immature rat uterus following E_2 treatment [36]. This dissociation might suggest that these 2 events are not casually linked.

Proto-oncogenes, however, are currently thought to be positive regulators of cell proliferation by enabling cells to traverse critical regulatory points in the cell cycle [37]. E_2 induced an accumulation of c-ras^H mRNA in the mouse uterine epithelium in a pattern similar to that described for other cell types [38,39]. In contrast to these other cell types however, the epithelial cells did not

Table 2

The activity of ornithine decarboxylase in the mouse uterine luminal epithelium following various hormonal treatments

Time following oestradiol-17β treatment (h)	Hormone treatment			
	Oestradiol-17β alone		Progesterone: oestradiol-17β	
	Enzyme activity	Fold stimulation	Enzyme activity	Fold stimulation
0	11 ± 3 (16)	–	54 ± 16 (4)[b]	4.9
2	18 ± 3 (4)[a]	1.6	84 ± 15 (4)[b]	7.6
4	232 ± 58 (10)[b]	21.1	390 ± 52 (4)[b]	35.5
6	653 ± 36 (4)[b]	59.4	832 ± 101 (4)[b]	75.6

The enzyme activity expressed as pmol/h per mg of protein ± SE of the mean was determined by the method of Djurhuus [26]. The fold stimulation is the degree of elevation of the enzyme activity observed at a specific time after E_2 treatment over that determined in the untreated ovariectomized luminal epithelium. The following degrees of significance were obtained when the activity from hormone-treated animals was compared to the untreated control: [a]not significant, [b]$p < 0.01$. The number in parentheses represents the number of observations

necessarily proceed to DNA synthesis and cell proliferation. It would also seem unlikely therefore that c-*ras*^{H} is a positive regulator of DNA synthesis acting independently of other controls, at least in the uterine luminal epithelium. Recent data has suggested that *ras* is a guanine nucloetide-binding protein [40] analogous to the G-protein known to regulate adenylate cyclase activity [41]. It may therefore act as a membrane signal transducer involved in preparing cells for division [42]. Perhaps this is achieved by stimulating the production of receptors to other growth factors to allow cells to progress towards DNA synthesis or by signalling for an elevated level of metabolic rate which is normally necessary for DNA synthesis initiation. Evidence favouring the latter role comes from the observation that progesterone treatment in the absence of E_2 produced a small increase in protein and rRNA synthesis [17] and c-*ras*^{H} mRNA but completely inhibited the basal level of cell proliferation [15,43]. Since in a normal epithelial tissue c-*ras*^{H} is not an obligatory cell cycle protein it is of considerable interest to understand how the function of a mutant *ras* protein [44,45] avoids the negative controls of other growth regulators and causes carcinomas in vivo.

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