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ESTROGEN INDUCTION OF INSULIN-LIKE GROWTH FACTORS AND myc PROTO-ONCOGENE EXPRESSION IN THE UTERUS

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Summary—In contrast to the effects observed *in vivo*, isolated uterine cells cultured *in vitro* demonstrate little proliferative response to estrogens. Estrogen induced uterine proliferation involves a carefully orchestrated, sequential activation of genes which encode a variety of biologically active molecules. These include nuclear transcription factors, growth factors and growth factor receptors. Expression of these proteins serve to amplify the effect of estrogen through cellular, autocrine and paracrine mechanisms. In this review the effects of estrogen on uterine expression of the myc family of oncogenes and the insulin-like growth factors are discussed.

INTRODUCTION

Virtually all cell types in the uterus demonstrate some degree of estrogen-responsiveness since marked atrophy of all layers of the uterus follows castration. Attempts to culture hormone responsive human and rodent uterine cells *in vitro* have been disappointing, since these cultures are either unresponsive or demonstrate a relatively poor growth response to physiological concentrations of estrogens [1–5]. This paradox suggests that the estrogen-induced mitogenic signal is in some way amplified or enhanced *in vivo*. A number of mechanisms may be responsible for the marked difference between the *in vivo* and *in vitro* responsiveness of uterine cells. One possible mechanism which has received some attention is the enhancement of estrogen action in individual cells by intracellular mediators and/or autocrine growth factors. However it is also possible that activation of paracrine growth factors and stromal–epithelial cell interactions are likely to be necessary for the uterine response to estrogen. Indeed, studies in the rodent have demonstrated that stromal–epithelial interaction is necessary for normal morphological development in the uterus during embryogenesis [6] and for estrogen-responsiveness of uterine epithelial cells in the neonatal mouse [7].

Studies from a number of laboratories have suggested that estrogen-induced uterine proliferation involves a carefully orchestrated, sequential activation of genes which encode a variety of biologically active molecules including; proto-oncogenes, *trans*-acting factors, growth factors, their receptors, chemotaxic factors and proteases (Fig. 1). In addition, estrogen-induced uterine proliferation may also involve the attenuation of expression of growth inhibitors. These events may occur in individual cells and thereby allow for expression of a factor or factors that act in an autocrine fashion. However, since the uterus is a complex organ composed of many different cell types, growth factors expressed in one cell may activate receptors in different cell populations and thus act in a paracrine fashion. In the immature rodent, cellular proliferation in response to estrogen can be documented in stromal and myometrial layers in addition to the epithelial layer. In the mature rodent, as is the case in the human, the epithelial layer appears to be most responsive to the cyclical changes in circulating estrogen levels [8]. Estrogen appears to have two effects on uterine epithelial cells in mature castrated animals [9, 10]. First, estrogen acts to recruit quiescent, G_0 cells into the cell cycle. In addition, estrogen accelerates cellular proliferation by shortening the G_1 phase. The effects of estrogen on myometrial and stromal cells are less well studied. While the effects of estrogen on these cell types are less dramatic in the adult rodent, both in the rodent and in man, castration results in atrophy of these layers.

A number of biologically important genes are activated in the uterus in response to estrogen

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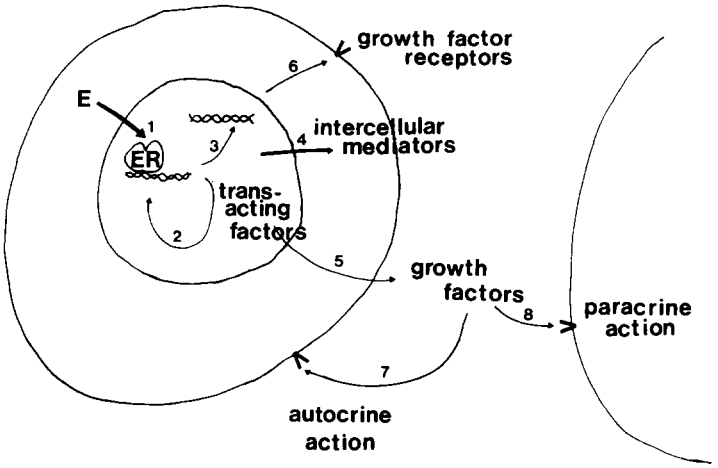

Fig. 1. Schematic plan of the multiple actions of estrogen on uterine cells. Estrogen (E) interacts with the estrogen receptor (ER) and the resulting complex interacts with EREs (1). Estrogen stimulates expression of a variety of genes including *trans*-acting factors (2) and intercellular mediators (4) which can stimulate expression of other genes (3) and may interact with genes containing EREs (2). Expression of a variety of growth factor (5) and growth factors receptors (6) is enhanced. These growth factors can interact in an autocrine fashion (7) or paracrine fashion (8).

and are of potential importance in estrogen-induced uterine proliferation. Examples of these genes are outlined in Table 1. In some circumstances, the estrogen response is apparent even when protein synthesis is inhibited suggesting that many of the actions of estrogen in the uterus may be direct. Interaction of estrogen with its receptor results in a complex which functions as a transcription enhancing factor by binding to *cis*-acting enhancer sequences usually located in the 5′ flanking region of the estrogen-responsive gene. Estrogen-responsive elements (EREs) have been identified in a number of genes and a considerable amount of effort has been directed towards identifying similar EREs in the estrogen-responsive genes listed in Table 1. However, as discussed below one of the earliest response demonstrable in the uterus following estrogen administration is enhanced expression of various proto-oncogenes which can also act as *trans*-acting factors. Thus, many of the demonstrable effects of estrogen on uterine gene transcription may not be a primary response to this hormone but rather a result of activation of other *trans*-acting factors. Estrogen-induced expression of an array of *trans*-acting factors would serve two purposes. Firstly, it would allow for an amplification of the initial signal. Even in genes where EREs may be present and where the initial effects of estrogen may be direct, activation of *trans*-acting factors other than the estrogen receptor may amplify the initial effect. Secondly, activation of a series of *trans*-acting factors may be a mechanism whereby estrogen can activate a pathway, common to mitogenic polypeptide growth factors and hormones and thereby stimulate uterine growth.

In this review I will discuss work from my laboratory concerning estrogen-induced uterine expression of the myc oncogenes and the insulin-like growth factors. The genes which encode these proteins represent examples of estrogen-responsive genes which are activated in the uterus. Space does not permit a detailed review of the many other functionally important genes which are expressed in the uterus in response to estrogen (Table 1).

Table 1. Genes expressed in the uterus in response to estrogen

Trans-acting factors	Growth factors and growth factor receptors	Other factors
c-jun	Epidermal growth factor	H-ras
c-fos	Epidermal growth factor receptor	Protease
c-myc	IGF-I	Chemotacic factors
Progesterone receptor	IGF-II	
	Type 1 IGF receptor	
	Colony stimulating factor	

ESTROGEN-INDUCED ACTIVATION OF myc PROTO-ONCOGENES

The myc proto-oncogenes encode nuclear proteins which appear to be intimately involved in the proliferative response of cells to a diverse array of stimuli. A rapid increase in expression of the c-myc proto-oncogene is seen in many quiescent cells following stimulation by mitogens. Although the functional role of the c-myc gene product has not been completely elucidated, microinjection of the c-myc protein [11] or transfection of the c-myc gene [12] "commit" quiescent fibroblasts to cellular division. The c-myc protein may be important in regulation of the transcriptional mechanism and appears to be one of a number of "commitment" proteins whose expression increases when quiescent cells are stimulated to proliferate. In many systems, expression of the c-myc gene appears to be necessary, but not of itself sufficient, to committed cells to enter the cell cycle. The c-myc proto-oncogene appears to be a member of a much larger gene family, to be described. Additional members of the myc oncogene family have been described. N-myc was first detected in a human neuroblastoma cell line [13]. Interestingly expression of N-myc shows a different temporal pattern in the developing mouse embryo and a different tissue distribution in the fetus and adult mouse to c-myc [14, 15]. L-myc is a c-myc related gene which was first detected in human small cell lung cancer. In the mouse, it appears to be expressed at significant levels only in fetal lung, brain and kidney and adult lung tissue [16].

Since the myc oncogenes demonstrate cell type specificity and respond rapidly to mitogen exposure we examined the possibility that expression of these genes may provide a useful tool to elucidate the mechanisms involved in the proliferative response of the uterus to estrogen [17]. We were the first group to report that expression of c-myc was estrogen-responsive. More recently other groups have demonstrated that c-myc expression is estrogen-responsive in estrogen receptor positive human breast cancer cells and that it is constitutively expressed in estrogen receptor negative breast cancer cell lines [18, 19].

An increase in c-myc mRNA abundance of approx. 4-fold was seen 1 h after E2 administration to ovariectomized rats, while peak expression, 9-fold relative to untreated ovariectomized rats, occurred 3 h after E2 (Fig. 2). In addition to the 2.4 kb c-myc transcript reported in other tissues, another less abundant 3.2 kb transcript was apparent at the time of maximal c-myc expression. Since the c-myc gene appears to have three functional promoters this additional transcript may represent transcription initiation at a more 5′ site. Significant induction of N-myc, approx. 6-fold compared to basal control levels, was apparent within 15 min of injection of E2. Maximal induction, 10-fold above basal levels occurred at 30 min (Fig. 2). The N-myc probe also hybridized to a 2.2 kb mRNA which demonstrated a different pattern of induction in the uterus after estrogen administration (Fig. 2). This may represent yet another member of the myc oncogene family. In contrast to the changes seen after estrogen administration to ovariectomized rats, little change in the abundance of the c-myc or N-myc transcripts in the uterus was seen throughout the estrous cycle. In the mature rat it is unlikely that there would be the synchronous recruitment of quiescent cells that is seen in the uterus of the immature or castrated rat after estrogen administration.

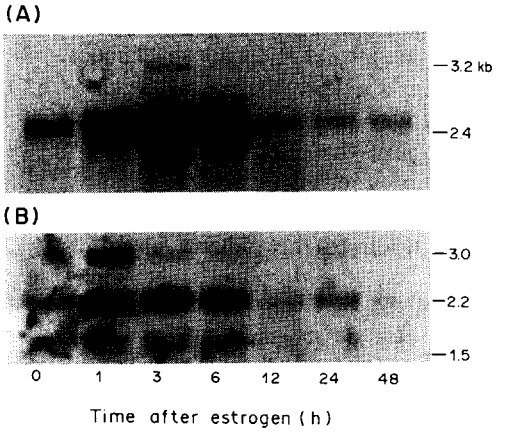

Fig. 2. Estrogen enhances uterine c-myc and N-myc mRNA abundance in the ovariectomized rat. Autoradiograms of Northern blots of uterine RNA from ovariectomized rats. Autoradiograms of Northern blots of uterine RNA from ovariectomized rats treated with estrogen are shown. In panel A the c-myc probe was used whereas the N-myc probe was used for hybridization in panel B.

In addition to activating the myc family of oncogenes, estrogen also stimulates expression of c-fos and c-jun in the rat uterus [20, 21]. The effect of estrogen on the expression of these *trans*-acting factors is not blocked by protein synthesis inhibitors and appears to be at the level of transcription. In addition, Weisz *et al.* [21], have shown that estrogen is able to elicit a response in MCF-7 mammary cancer

cells transfected with a reporter plasmid containing the jun/AP-1-response element. These data if applicable to the uterus suggest that the initial activation of certain *trans*-acting factors may amplify the effects of estrogen on gene expression by indirectly activating transcription of other genes which lack EREs.

In addition to the indirect amplification of estrogen-response on gene expression some recent data suggests that the jun/fos *trans*-acting factors may be able to interact directly with steroid hormone receptors and hormone-responsive elements [22]. Diamond *et al.* [22] have shown that regulatory function of the composite glucocorticoid-response element in the mouse proliferin gene is dependent upon the presence or absence of the jun/fos proteins. In their studies the glucocorticoid-response element was inactive in the absence of c-jun, conferred a positive glucocorticoid-response in the presence of c-jun and a negative glucocorticoid effect in the presence of both jun and fos. If similar interactions occur with the estrogen receptor and EREs, it is possible that this may be yet another mechanism whereby the effects of estrogen are amplified, attenuated or modulated.

The initial studies on the myc and fos oncogenes focused on the effects of polypeptide growth factors, e.g. platelet-derived growth factor, on expression of these oncogenes in quiescent fibroblasts [12, 23]. It is now clear that a wide variety of agents in addition to polypeptide growth factors can also activate expression of these genes. Although there is considerable evidence that some of the effects of estrogen on cellular proliferation, particularly mammary cell growth, are mediated by the production of polypeptide growth factors [24], it appears that even in human breast cancer cells the effect of estrogen on c-fos and c-myc can be independent of production of growth factors [25]. That is estrogen can directly activate these genes. However, the possibility remains that the subsequent estrogen-induced synthesis of a variety of growth factors may amplify the estrogen-response. This amplification of estrogen-response may occur via an autocrine loop if the appropriate growth factor receptors are present. However in a complex tissue such as the uterus paracrine interactions may also occur.

Uterine tissue contains a variety of growth factors some of which have not as yet been fully characterized [26, 30]. Epidermal growth factor and the insulin-like growth factor have received most attention to date. Each of these appears to have some role to play in the estrogen-induced uterine proliferative-response.

THE INSULIN-LIKE GROWTH FACTORS (IGFs)

In the rodent both IGF-I and IGF-II are expressed in the uterus and expression of these growth factors is up-regulated by estrogen. In the mature ovariectomized rat, a single injection of 17β estradiol results in a 10-fold increase in uterine IGF-I mRNA abundance [26]. The increase in IGF-I mRNA reached a maximum at 6 h after injection and then gradually declined towards basal level (Fig. 3). A qualitatively and quantitatively similar response is demonstrable in the immature rat. Repeated injections of estrogen result in an equally rapid response as that seen after the initial injection. When immature rats are subjected to daily injections of 17β estradiol there is a gradual decrease in the

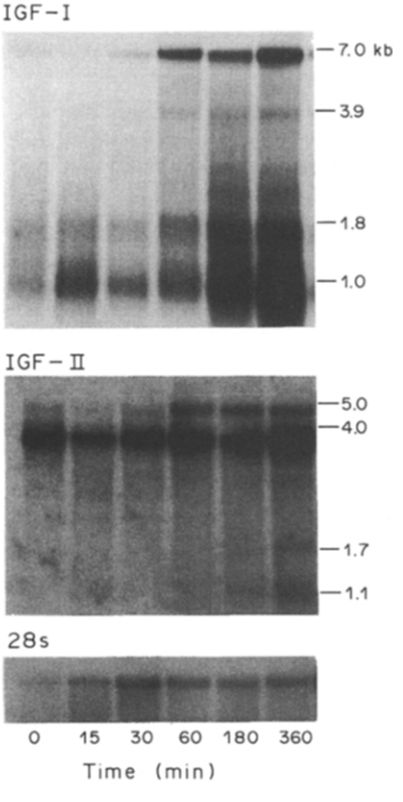

Fig. 3. Estrogen enhances uterine IGF-I and II mRNA abundance in the ovariectomized rat. Autoradiograms of a Northern blot of RNA from ovariectomized rats treated with estrogen are shown. In the upper panel rat IGF-I cDNA has been used as a hybridization probe. In the middle panel a mouse IGF-II cDNA has been used as a hybridization probe while in the lower panel the filter has been hybridized a 28s ribosomal probe as a control for gel loading. Reproduced with permission from Ref. [33].

maximal IGF-I response and a gradual rise in the basal levels of IGF-I mRNA detected immediately prior to each injection [31]. The uterine IGF-I response is direct, in that, it does not require continuing protein synthesis [32]. This estrogen-response also appears to be pituitary-independent and a response of equal or greater magnitude is seen in hypopituitary animals [31]. Uterine IGF-II mRNA abundance also increases in response to estrogen administration to ovariectomized rats however the increase is considerably more modest than in the case of IGF-I (Fig. 3). Similarly, an increase in uterine IGF-I mRNA is seen in immature rats injected with estrogen. In contrast to the dramatic response in the immature animal and castrated mature rat, there is only a very small variation in the abundance of IGF-I throughout the estrous cycle [33]. This lack of obvious estrogen-dependence in the mature animal may result from the fact that most of the uterine IGF-I mRNA appears to be localized to the myometrium [34]. As discussed above, the myometrium shows little proliferative response to the cyclic variations in estrogen levels in the mature intact rat but does proliferate in response to estrogen in the estrogen naive immature rat or the castrated mature rat [8–10, 35].

In addition to the increase in uterine IGF-I mRNA which is seen following estrogen administration to the castrated rat, an increase in acid-ethanol extractable, immunoreactive IGF-I can also be demonstrated. It is not clear what proportion of this increase is due to local uterine IGF-I synthesis. The increased uterine blood flow consequent to estrogen administration may be responsible for increased delivery and uptake of circulating IGF-I. It is however possible to detect preprolGF-I by immunoprecipitation of biosynthetically labelled uterine proteins. This suggests that at least some, if not all, of the uterine IGF-I mRNA can be translated [36].

Both IGF-I and II mRNA are detectable in human endometrial biopsies' tissue with the abundance of IGF-II mRNA being considerably lower than that of IGF-I. The abundance of IGF-I mRNA in endometrial biopsies from the secretory and proliferative phases of the menstrual cycle is similar [37]. IGF-I and II mRNAs are particularly abundant in human uterine leiomyomata [38] suggesting that, as in the rat, these growth factors may be expressed predominantly in uterine smooth muscle cells. In the rat, *in situ* hybridization localizes the IGF-I mRNA to the stromal and myometrial cells with little expression in the luminal epithelium [34, 39]. In the stroma, IGF-I mRNA appears to be particularly abundant in the basal, perimyometrial or outer layer and in the periglandular stroma [39]. In addition, when epithelial, stromal and myometrial layers of mature intact rat uteri are prepared by mechanical and enzymatic methods approx. 4, 35 and 61 of the total uterine IGF-I mRNA is present in each of these three layers, respectively [17].

IGF-1 RECEPTORS AND BINDING PROTEINS

Receptors for IGF-1 have been demonstrated in rat uterine tissue. The affinity and capacity ($K_d = 0.12$ nM, $B_{max} = 0.028$ pmol/mg protein) of these receptors are similar to that reported for IGF receptors in other tissues [40]. Affinity cross-linking experiments with ^{125}I-IGF-I demonstrated that the IGF-I receptor is of the type-1 variety with an alpha subunit of approx. 135 kDa. It is possible to demonstrate autoradiographically that the majority of ^{125}I-IGF-I binding is localized to the smooth muscle cells of the myometrial layer of the uterus, particularly the outer longitudinal smooth muscle layer [40]. The co-localization of both IGF-I and IGF-I receptors to the myometrial layer of the uterus supports the concept that IGF-I may function as an autocrine "estromedin" in this tissue. However, the autoradiographic techniques used to localize IGF-I receptors in the uterus are relatively insensitive. It is likely that these receptors are also present in uterine epithelial and stromal cells albeit at the lower level.

Uterine IGF-I receptors appear to be under similar hormonal control to uterine IGF-I expression. In both immature and ovariectomized rats, administration of estradiol increases the number of uterine IGF-I receptors with no significant effect on receptor affinity [40]. However, compared to the effect of estradiol on other uterine proteins, the up-regulation of IGF receptors is quite modest. In mature cycling rats, the total uterine ^{125}I-IGF-I binding capacity was greatest during proestrus and the lowest ^{125}I-IGF-I binding was seen in the diestrous uteri. As discussed above, only a small variation in uterine IGF-I mRNA is seen during the estrous cycle with highest levels late in diestrus and early in proestrus [33]. It is not clear whether the effect of estrogen on uterine IGF receptors is direct or secondary to up-regulation of IGF-I expression or other estrogen-induced responses.

In addition to the type 1, IGF receptor, affinity cross-linking and ligand blotting experiments have demonstrated that at least four distinct binding proteins are present in uterine extracts and luminal fluid [36]. In ligand blotting experiments of uterine luminal fluid it is possible to demonstrate proteins of 42, 40, 34, 30, 27 and 24 kDa which bind ^{125}I-IGF-I. The precise relationship of these IGF binding proteins to the binding proteins which have so far been isolated is not clear. Using DNA and immunological probes we have demonstrated that IGFBP-1 and 3 are expressed in uterine tissue. These binding proteins are thought to regulate the bioavailability and therefore the action of the IGFs. The consensus view is that the IGF binding proteins are able to inhibit the biological actions of the IGFs [41–44]. However, this is not a uniform finding and at least under some circumstances, IGFBP-1 can actually enhance the action of IGF-I [45]. It is of note that IGF-I variants which do not bind to the binding protein are considerably more potent that IGF-I in a variety of cell culture systems [46].

In the rat, immunohistochemistry and *in situ* hybridization localize IGFBP-1 immunoreactivity and IGFBP-1 mRNA to the uterine luminal epithelium and the stromal glandular tissue. In the immature rat, estrogen administration results in a marked decrease in uterine IGFBP-1 mRNA abundance and in the mature rat the levels of IGFBP-1 are highest in diestrus and lowest in proestrus [36]. If IGFBP-1 actually inhibits IGF-I action in the uterine tissue, then the attenuation of expression of this binding protein may increase the bioavailability and functional activity of uterine IGF-I.

In organ culture, IGF-I amplifies estrogen-induced uterine DNA synthesis in rat uterine sections [34]. This response is most marked in the presence of estrogen and in tissue derived from estrogen-pretreated rats [34]. These data suggest that IGF-I is able to function in a autocrine/paracrine fashion in the uterus. This paracrine loop can be modulated by estrogen at least at two levels; enhanced expression of the ligand, IGF-I and increased abundance of the receptor for this growth factor. However the paracrine action of the IGFs in uterine tissue may be considerably more complex since at least three different IGF binding proteins are also present in uterine fluid and in uterine extracts.

CONCLUSIONS

The recruitment of growth arrested quiescent cells involves a careful orchestrated sequence of events. In the model originally proposed by Stiles *et al.* [47] whereby serum-deprived mouse fibroblasts are recruited from the G_0/G_1 phase into the cell cycle, two distinct stages were identified. Initial transient exposure of the quiescent cells to certain polypeptide growth factors resulted in the commitment of these cells to division providing appropriate factors were present to allow for progression through the G_0/G_1 phase to the S-phase of the cell cycle. In their system platelet-derived growth factor was a potent commitment factor while IGF-I was required for progression. Although rather simplistic, this model is conceptually useful in understanding the events involved in cellular proliferation. It has since been recognized in a number of systems that c-myc and c-fos activation are markers of the commitment. Activation of these genes is necessary but not sufficient to allow for cellular proliferation. With certain limitations and modifications, this rather simplistic model can be applied to whole organs composed of multiple cell types. For example, stimulation of hepatic cell proliferation in hypopituitary rats is accompanied by a sequential activation of c-myc and IGF-I expression [48]. Similarly, in the uterus estrogen-induced growth is accompanied by the same sequence of events. In these tissues, an added complexity to the commitment/progression model is that certain factors produced by one cell type may be able to function in a paracrine fashion on adjacent cells. In the uterus, estrogen appears to act in two ways to bring about cellular proliferation. In addition to recruiting cells out of the G_0/G_1 phase into the cell cycle, it also acts to shorten the G_1 to S transition presumably by providing the appropriate progression factors. While this review has focused on the myc and IGF family of genes clearly these proteins serve only as examples of different factors which are activated by estrogen. It is not clear whether activation of either of these groups of genes is either sufficient or necessary for estrogen-induced uterine proliferation.

We and other investigators have focused attention on identifying EREs in genes like IGF-I which appear to be estrogen inducible in the uterus. However since estrogen activates a variety of *trans*-activating factors, a rapid increase in transcription of a vast variety of genes may

be possible through this indirect mechanism. Clearly, this mechanism could amplify direct estrogen responses and allow a wide variety of stimuli to mediate a common final pathway which results in cellular proliferation.

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