EXPERIMENTAL STUDY

Potentiation response of cultured human uterine leiomyoma cells to various growth factors by endothelin-1: role of protein kinase C

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Abstract

Objective: Factors responsible for the abnormal proliferation of myometrial cells that accompanies leiomyoma formation are unknown, although steroid hormones and peptide growth factors have been implicated. We hypothesized that endothelin-1 (ET-1) is a physiological regulator of tumor growth.

Design: In this study, we investigated the role of ET-1 on growth of human leiomyoma cells and its synergistic effect with growth factors, as well as the signaling pathway involved in this interaction.

Methods: Leiomyoma cell proliferation was assayed by [3H]thymidine incorporation and cell number. Protein kinase C (PKC) isoforms were analyzed by Western blot using specific antibodies.

Results: ET-1 on its own was unable to stimulate DNA synthesis but potentiated the leiomyoma cell growth effects of basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), IGF-I and IGF-II. The failure of a protein tyrosine kinase (PTK) inhibitor, tyrphostin 51, to affect the potentiating effect of ET-1, supports the hypothesis of non-involvement of PTK in this process. The inhibition of PKC by calphostin C or its down-regulation by phorbol 12,13-dibutyrate (PDB) eliminated the potentiating effect of ET-1, but did not block cell proliferation induced by the growth factors alone. Five PKC isoforms (α, β1, ε, δ and ζ) were detected in leiomyoma cells, but only phorbol ester-sensitive PKC isoforms (PKCα, ε and δ) contribute to the potentiating effect of leiomyoma cell growth by ET-1.

Conclusions: We have demonstrated that ET-1 potentiates leiomyoma cell proliferation to growth factors through a PKC-dependent pathway. These findings suggest a possible involvement of ET-1 in the pathogenesis of leiomyomas.

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Introduction

Uterine leiomyomas are benign smooth muscle tumors of the myometrium arising in as many as 30% of women over 35 years old. Leiomyomas are the commonest indication for hysterectomy due to menorrhagia and pelvic pain. Factors responsible for enlargement of leiomyomas have been poorly understood. Steroid hormones are strongly implicated because these tumors appear during the reproductive years, increase in size during pregnancy and stabilize or regress after menopause. Traditionally, estrogen has been considered as the major promoter of leiomyoma growth, but clinical observations also suggest that progesterone plays a critical role in the pathogenesis of uterine leiomyomas (for a review see ref. 1). The mitogenic effects of estrogens are likely mediated by local production of growth factors and their receptors (2, 3). Leiomyoma cells produce more of several growth factors such as epidermal growth factor (EGF), insulin-like growth factors (IGF-I, IGF-II) and basic fibroblast growth factor (bFGF) than do the normal myometrial cells and more of their corresponding receptors (4–8). Attention has recently been drawn to the possibility that endothelin-1 (ET-1) might be a promoter of myometrial cell growth because this peptide may be involved in the development of a variety of neoplasms, acting in an autocrine/paracrine fashion during tumor development and metastasis (9). The exclusive presence of ET A receptors functionally coupled to phospholipase C in leiomyomas (10–12), together with the establishment of ET-1 growth properties in cultured human myometrial cells (13), led to the postulation that ET-1 might be a new regulator of the growth of leiomyomas.
Despite accumulating evidence of the pathophysiolo-
gical significance of ET-1 as a mitogen, there is still no
certainty that this peptide has growth properties in
human leiomyoma cells. Conventionally, calcium, pro-
tein kinase C (PKC) and protein tyrosine kinase
(PTK) signaling pathways are thought to contribute to
ET-1-induced cell growth and differentiation. However,
in normal human myometrial cells, substantial evi-
dence indicates that only the activation of PKC is
required for ET-1-induced proliferation (14). PKCs
constitute an expanding multigene family, so far with
12 known isoforms classified into three subfamilies on
the basis of their structure and ability to bind co-

factors. These are: the conventional PKC isoforms (α,
β1, β2, γ), which can be activated by calcium and
diacylglycerol (DAG) or phorbol esters; the novel PKC
isoforms (δ, ε, η, θ), which can be activated by DAG and
phorbol esters, but in a calcium-independent manner;
and the atypical PKC isoforms (ζ, λ, η). This last group is
unresponsive to calcium and DAG or phorbol esters, but
is regulated by other phospholipidic mediators (for a
review see ref. 15). However, it was not then possible to
say whether PKC is responsible for growth factor-
induced proliferation in cultured leiomyoma cells, or is
necessary and/or sufficient for the synergistic effect of
ET-1 with growth factors.

Accordingly, the first aim of this study was to
determine whether ET-1 by itself is mitogenic for
cultured human leiomyoma cells, or if it acts as a co-
mitogen with growth factors to induce DNA synthesis
and cell proliferation. The second aim of the study was
to examine whether PKC and PTK are coupled to
downstream signaling pathways that regulate lei-
omyoma cell growth by ET-1.

Materials and methods

Chemicals

Dulbecco’s Modified Eagle’s Medium (DMEM) with or
without phenol red and fetal calf serum (FCS) were
from GIBCO Life Technologies (Cergy, Pontoise, France).
[methyl-3H]thymidine (50 Ci/mmol), Hybond-C mem-
branes, the enhanced chemiluminescence detection
system (ECL), and X-rays films were obtained from
Amersham International (Chalfont, Buckinghamshire,
UK). ET-1, sarafotoxin 6c (S6c) were from Neosystem
Amersham International (Chalfont, Buckinghamshire,
USA). They were raised in rabbits against peptides
corresponding to amino acid sequences 656–671 and 656–673 respec-
tively. The second antibody, donkey anti-rabbit IgG
conjugated to horseradish peroxidase, was purchased
from Amersham International, and pre-stained
molecular-weight markers were from Bio-Rad (Rich-
mond, CA, USA). EGF, IGF-I, IGF-II and bFGF were
purchased from Chemicon International (Temecula,
CA, USA). Phorbol 12,13-dibutylate (PDB), leupeptin,
Nonidet P-40, phenylmethylsulfonylfluoride (PMSF),
calphostin C, tyrphostin 51, and other drugs and
chemicals used were of the highest quality available
from Sigma.

Leiomyoma cell culture

Tissue specimens were obtained from four cycling
women aged 39–45 years undergoing hysterectomy
for symptomatic uterine leiomyomas. All women were
in the follicular phase of the menstrual cycle. None had
received hormone treatment for at least 3 months
before surgery. The uteri were examined by a pathol-
gist to exclude adenomyosis or malignant change. The
samples of intramural leiomyoma were all of similar
size (2 cm diameter) to standardize the experimental
protocol. To avoid contamination with endometrium,
no submucosal leiomyoma was collected. After collect-
ion, the samples of leiomyoma were placed in DMEM
supplemented with 100 IU/ml penicillin and 100 µg/
ml streptomycin. This study was approved by the
Comité Consultatif de Protection des Personnes pour la
Recherche Biomédicale (Paris-Cochin, France).

Leiomyoma cells were prepared using the explant
method. Cells were cultured in DMEM supplemented
with antibiotics and 10% FCS. For cell growth
experiments, leiomyoma cells were used between
passages 3 and 6, with no noticeable difference in
results observed with cells from individual passages
or with cells isolated from different uteri. Cultured
leiomyoma cells were identified by their positive
reaction with monoclonal antibodies against smooth
muscle α-actin, smooth muscle-I (SM1) and SM2
myosin heavy chains and by the typical ‘hill and valley’
microscopic findings. Each population of leiomyoma
cells studied came from a separate patient.

Assessment of proliferation

Leiomyoma cells (3000–4000 cells/well) were cultured
to subconfluence in 96-well dishes in the presence of
10% FCS for 48 h. The cells in exponential growth
were then transferred to serum-deprived media for
72 h to achieve quiescence. Quiescent cells were
incubated for 48 h in serum-free media in the presence
of various concentrations of ET-1 and/or growth
factors, as previously described (13). In combination
experiments, protein kinase inhibitors were added
30 min before incubation with ET-1 and/or growth
factors. None of these drugs tested was toxic to human
leiomyoma cells under our conditions as controlled by
trypan blue exclusion. Serum-free DMEM was used as a
negative control and DMEM with 10% FCS as a positive
control. [3H]thymidine (0.4 µCi/well) was added during
the final 24 h of incubation. After incubation, cells were washed twice with phosphate-buffered saline (PBS) without Mg$^{2+}$ and Ca$^{2+}$, fixed with 5% trichloroacetic acid, washed twice with 100% ethanol, and solubilized with 0.5 N sodium hydroxide. Cell-associated radioactivity was measured by scintillation counting. Cell numbers were determined in separate experiments. Quiescent human leiomyoma cells were incubated with similar treatments. After 72 h, cells were trypsinized and counted with a hemocytometer. The viability of cells was controlled by trypan blue staining.

Western blot analysis of PKC
The experiments were carried out as previously described with cultured human myometrial cells (14). Subconfluent leiomyoma cells were left untreated or treated with 0.1 μmol/l PDB for 48 h in serum-free medium and subsequently washed twice with PBS. The cells were scraped into 20 mmol/l Tris–HCl buffer, pH 7.5, containing 250 mmol/l sucrose, 1 mmol/l EGTA, 2 mmol/l EDTA, 50 mmol/l β-mercaptoethanol, 2 mmol/l PMSF, 5% glycerol, and 40 μg/ml leupeptin and were sonicated twice for 10 s. Equal amounts of protein lysates (40 μg) were separated by 8% SDS-PAGE and after electrophoretic separation, proteins were transferred to a nitrocellulose membrane. Non-specific binding sites were blocked by incubating the membrane with 5% fat-free dried milk in TBST (10 mmol/l Tris–HCl, pH 7.5, 0.15 mol/l NaCl, 0.1% Tween 20). Anti-PKC antibodies were added at the appropriate concentration and incubated for 45 min at room temperature. The membrane was washed with TBST and incubated for 30 min with the secondary antibody. The blots were developed with ECL reagents and visualized on Kodak X-ray films, and the intensities of the immunoreactive bands were analyzed densitometrically by the NIH Image 1.60 Software Package (NIH, Bethesda, MD, USA). The specificity of the immunobands was tested by competition with appropriate peptides used as immunogens.

Statistical analysis
Data are shown as means ± s.e. We used the two-tailed Wilcoxon paired nonparametric signed-rank test to analyze the data from the five to ten separate experiments. A P value <0.05 was accepted as significant.

Results

Effect of ET-1 on DNA synthesis and proliferation of cultured leiomyoma cells
ET-1 alone (up to 1 μmol/l) did not significantly increase leiomyoma cell number (Fig. 1A) or [3H]thymidine incorporation, whereas bFGF, EGF, IGF-I or IGF-II (15 nmol/l) caused leiomyoma cell proliferation, with increases in DNA synthesis of 179 ± 11%, 147 ± 11%, 168 ± 14% or 149 ± 12% of the serum-free control respectively (Fig. 1B). The increase in cell number in response to bFGF, EGF, IGF-I or IGF-II (15 nmol/l) was potentiated by ET-1 (0.1 nmol/l to 1 μmol/l) in a concentration-dependent manner (Fig. 1A). Similarly, the increase in DNA synthesis in response to co-addition of ET-1 (100 nmol/l) with each growth factor (15 nmol/l) to leiomyoma cells resulted in maximal stimulation of 237 ± 8% (bFGF), 144 ± 11% (EGF), 144 ± 11% (IGF-I) or 159 ± 12% (IGF-II) (Fig. 1B).

![Figure 1](https://www.eje.org/)

**Figure 1 (A)** Effect of increasing concentrations of ET-1 on leiomyoma cell proliferation with and without growth factors. Quiescent leiomyoma cells (4000 cells/well) were incubated with the indicated concentrations of ET-1 without (●) or with 15 nmol/l bFGF (●), EGF (●), IGF-I (●), or IGF-II (●). This experiment was repeated twice independently. (B) [3H]Thymidine incorporation induced by 100 nmol/l ET-1 and/or bFGF, EGF, IGF-I or IGF-II (15 nmol/l) and were determined as in Materials and Methods and are expressed as a percentage of serum-free control. Basal [3H]thymidine incorporation was 6802 ± 964 d.p.m./well. Data are means ± s.e. of quadruplicate determinations from 5–17 independent experiments, each performed on a different population of leiomyoma cells (subcultures 3–6) isolated from four different uteri. *P < 0.05 and **P < 0.001 as compared with serum-deprived cells. †P < 0.05 as compared with growth factor alone.
significantly increase DNA synthesis beyond that obtained with the growth factor alone, however the levels of \([^3]H\)thymidine incorporation were consistently greater than those in serum-free conditions. Comparable results were obtained with co-application of ET-1 and IGF-II. When exposed to Tyr 51, leiomyoma cells lost the responsiveness to the mitogenic activity of co-addition of ET-1 and EGF or IGF-I. All these results suggest that treatment of leiomyoma cells with Tyr 51 prevents the potentiating effect of ET-1.

We next examined the effect of calphostin C, a PKC inhibitor, to assess the contribution of PKC to ET-1-potentiated leiomyoma cell proliferation with growth factors. Calphostin C has a dose-dependent inhibitory effect on cell proliferation induced by ET-1 (100 nmol/l) with each of the growth factors tested (Fig. 2A). Pretreatment of cells with 100 nmol/l calphostin C significantly attenuated the enhanced DNA synthesis induced by a combination of ET-1 and growth factors. By contrast, calphostin C did not significantly decrease \([^3]H\)thymidine incorporation induced by bFGF, EGF, IGF-I or IGF-II alone (Fig. 2B). The role of PKC on the potentiating effect of ET-1 on cell proliferation was further investigated by examining the effects of PKC down-regulation on this response. Leiomyoma cells were pretreated for 72 h with 100 nmol/l PDB to deplete phorbol ester-sensitive PKC isoforms. The PDB treatment did not change the basal leiomyoma cell number. Whereas the cell proliferation induced by growth factor alone was unaffected by PDB treatment (data not shown), the increase in cell number induced by simultaneous addition of ET-1 and growth factors was reduced and returned to level obtained with the growth factor alone (Fig. 3A). We next investigated which phorbol ester-sensitive PKC isoforms are present in leiomyoma cells. Western blot analysis revealed the presence of at least five PKC isoforms: PKCa, PKCb1, PKCb6, PKCε and PKCe (Fig. 3B). Long term-treatment with 100 nmol/l PDB

| Table 1 Effect of PTK inhibition by tyrphostin 51 on the potentiation of growth factors by ET-1. Results are expressed as a percentage of serum-free control (100%). Data are means ± S.E. |
|-----------------|-----------------|-----------------|
| None | Tyrphostin 51 |
| ET-1 | 107 ± 15 | 102 ± 13 |
| bFGF | 139 ± 14* | 113 ± 11 |
| bFGF+ET-1 | 181 ± 13† | 133 ± 12* |
| EGF | 138 ± 17* | 108 ± 7 |
| EGF+ET-1 | 192 ± 18† | 110 ± 13 |
| IGF-I | 139 ± 15* | 112 ± 11 |
| IGF-I+ET-1 | 177 ± 15† | 110 ± 7 |
| IGF-II | 133 ± 12* | 114 ± 8 |
| IGF-II+ET-1 | 170 ± 14† | 133 ± 10* |

*P < 0.05 vs control, †P < 0.05 vs growth factor alone.

234 ± 11% (EGF), 221 ± 14% (IGF-I) and 212 ± 15% (IGF-II) of the serum-free control (Fig. 1B).

Under the same conditions, the receptor ETB agonist, sarafotoxin 6c (S6c) (0.1 nmol/l–1 ET-1 and bFGF, under Tyr 51 treatment, did not increase in \([^3]H\)thymidine incorporation induced by bFGF, EGF, IGF-I or IGF-II. When exposed to Tyr 51, leiomyoma cells lost the responsiveness to the mitogenic activity of co-addition of ET-1 and EGF or IGF-I. All these results suggest that treatment of leiomyoma cells with Tyr 51 prevents the potentiating effect of ET-1.

Effect of PTK and PKC inhibition on the potentiating effect of ET-1

To further evaluate whether PTK activation was required for mitogenic signaling by both ET-1 and growth factors, we examined the effect of tyrphostin 51 (Tyr 51), the most potent EGF-receptor kinase inhibitor. We confirmed that in the presence of Tyr 51, none of the growth factors bFGF, EGF, IGF-I or IGF-II was able to induce a significant proliferative effect. At a concentration of 100 nmol/l, Tyr 51 reduced by 78–86% the increase in \([^3]H\)thymidine incorporation induced by 15 nmol/l of growth factor (Table 1). The co-addition of ET-1 and bFGF, under Tyr 51 treatment, did not

Figure 2 Effect of PKC inhibition by calphostin C on the potentiation of growth factors by ET-1. (A) Quiescent leiomyoma cells (4000 cells/well) were pre-incubated with increasing concentrations of calphostin C for 30 min before adding 100 nmol/l ET-1 with 15 nmol/l bFGF (●), EGF (○), IGF-I (□) or IGF-II (▲). Data shown are percentage of maximal cell number increase observed with ET-1 and growth factors and are from a single experiment representative of the three giving similar results (subcultures 3–5). (B) Effect of 100 nmol/l calphostin C on 15 nmol/l growth factor-induced increase in \([^3]H\)thymidine incorporation. Results are expressed as percentage of serum-free control. Data are means ± s.e. of quadruplicate determinations from four independent experiments, each performed on a different population of leiomyoma cells (subcultures 3–6) isolated from two different uteri.
induced a decrease in the total immunoreactive amounts of PKCα, PKCδ and PKCε but not PKCβ1 and PKCζ. The isoforms PKCα and PKCδ were the most sensitive as 45% and 49% were respectively lost after this treatment. Under the same conditions, PKCε immunoreactivity was decreased by only 25%.

**Discussion**

The present study demonstrated that ET-1 potentiated the proliferative effect of growth factors through a PKC-dependent pathway in cultured human leiomyoma cells.

Whereas ET-1 on its own is not mitogenic, this peptide is an effective co-mitogen with polypeptide growth factors such as bFGF, EGF, IGF-I and IGF-II in leiomyoma cells. This contrasts with the absence of a requirement for growth factors to elicit an ET-1-induced growth response in normal human myometrial cells grown in culture (13). A question arises over the role of specific autocrine growth factors in leiomyoma cell proliferation. An abnormal elevated expression of different growth factors and/or their receptors has been demonstrated to be involved in the pathogenesis of leiomyomas. Thus, leiomyoma cells are a more important endogenous source of bFGF, EGF, IGF-I and IGF-II than normal myometrial cells (4–8). As expected, exogenous bFGF, EGF and IGFs stimulated the DNA synthesis and proliferation of leiomyoma cells. Synergism between ET-1 and growth factor effects is a common phenomenon in vascular smooth muscle. Numerous investigations have revealed that ET-1 is either not at all a proliferative agent or only a very weak proliferative one but that it potentiates PDGF-dependent (platelet-derived growth factor-) EGF- and bFGF-induced mitogenic effect in human vascular smooth muscle cells (16–18).

In addition, the failure of the selective ETβ agonist S6c to initiate DNA synthesis in the absence or presence of growth factors argues against an ETB receptor being linked to these proliferative responses. Although further experiments are necessary to confirm the presence of ETA receptors in cultured leiomyoma cells, our findings agree with our recent results that demonstrated functional ETA and ETB receptors in normal human myometrium, while only functional ETA receptors were detected in human uterine leiomyomas (11, 12). In contrast to peptide growth factor receptors, which possess intrinsic protein tyrosine kinase activities, G-protein coupled receptors (GPCRs), such as ETA receptors, have not been demonstrated to possess endogenous tyrosine kinase. Next, we have investigated which PTK or PKC pathway was responsible for cooperative action between ET-1 and growth factors to induce leiomyoma cell proliferation. Tyrosine kinase activation may be sufficient for growth factors to stimulate proliferation of cultured leiomyoma cells, whereas the potentiating effect of ET-1 appears to require additional signals. In addition, our present data indicate that the mitogenic response of leiomyoma cells to bFGF, EGF and IGFs is not dependent on PKC activation. Neither [3H]thymidine incorporation nor the cell number increase in response to each growth factor was significantly blocked by PKC inhibition. By contrast, most of the potentiating effect of ET-1 was clearly inhibited by the PKC inhibitor, calphostin C. The loss of the ET-1 effect when co-administered with bFGF, EGF and IGFs in PKC-depleted leiomyoma cells obtained after prolonged treatment with PDB further supports a crucial role for phorbol ester-sensitive isoforms of PKC in this process. We next identified the pattern of expression of PKC isoforms in cultured human leiomyoma cells. Among the five isoforms of PKC (α, β1, ε, δ and ζ) found in uterine leiomyoma cells, we believe that either PKCα, PKCδ and PKCζ
alone or in combination, rather than atypical phorbol ester-insensitive PKC\(\varepsilon\), are involved in the potentiating effect of ET-1. However, the surprising resistance of PKC\(\beta\) to being down-regulated by PDB in cultured leiomyoma cells supports the hypothesis that this isoform should not be required for eliciting potentiating effect of ET-1. The reason for the retention of membrane PKC\(\beta\) in PDB-down-regulated leiomyoma cells is not presently clear, and caution must be exercised in interpreting such data. We cannot rule out the possibility that prolonged treatment by a phorbol ester might result in nuclear localization of this isoform, without any down-regulation as previously reported in human T-cells (19). The existence of multiple PKC isozymes in leiomyoma cells may reflect the various specialized cellular functions mediated by this kinase (20). However, although the roles of individual isozymes in migration, invasion and in growth control have only recently begun to be explored, the type of isoform involved appears to depend on the cell type (21). By identifying the specific role(s) of each of these PKC isoforms, insights may be gained into how ET-1 regulates leiomyoma cell proliferation, and how it contributes to leiomyoma formation. Further work is required to identify the signaling pathway downstream of PKC in leiomyoma cells.

Taken together, our data have demonstrated a growth-potentiating effect of ET-1 in human uterine leiomyoma cells in response to growth factors through a PKC-dependent pathway. It is likely that ET-1 acts in an autocrine–paracrine fashion as a mediator of growth factors in promoting tumor enlargement. Whether this mechanism has any part to play in the pathogenesis of leiomyomas, remains to be determined.

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References

16 Hafizi S, Allen SP, Goodwin AT, Chester AH & Yacoub MH. Endothelin-1 stimulates proliferation of human coronary smooth muscle cells via the ET\(\alpha\) receptor and is co-mitogenic with growth factors. Atherosclerosis 1999 146 351–359.
17 Weissberg PL, Witchell C, Davenport AP, Hesketh TR & Metcalfe JC. The endothelin peptides ET-1, ET-2, ET-3 and sarafotoxin S6b are co-mitogenic with platelet-derived growth factor for vascular smooth muscle cells. Atherosclerosis 1990 85 257–262.

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