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Estrogen Induces c-*Ha-ras* Expression Via Activation of Tyrosine Kinase in Uterine Endometrial Fibroblasts and Cancer Cells

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Endometrial fibroblasts derived from uterine endometrium as controls and endometrial cancer cells (Ishikawa and HHUA cells) were used to analyze the manner of induction of c-*Ha-ras* transcripts in endometrial cancers, some of which are estrogen-dependent in growth. Estrogen increased c-*Ha-ras* expression and tyrosine kinase (TK) activity in fibroblast and Ishikawa cells, but not in HHUA cells. Progesterone diminished c-*Ha-ras* expression and tyrosine kinase (TK) activity induced by estradiol in the fibroblasts, but not in Ishikawa cells, which persistently overexpressed c-*Ha-ras*. In these cells, epidermal growth factor (EGF) increased c-*Ha-ras* expression as did estradiol. Pretreatment with tyrphostin, an inhibitor of TK, abolished estrogen-inducible overexpression of c-*Ha-ras*. The combination of both estradiol and EGF at maximum effective concentration exerted no additive or synergistic effect on induction of c-*Ha-ras* expression. In conclusion, persistent activation of TK might lead to overexpression of c-*Ha-ras* in some endometrial cancer cells under estrogen predominant milieu, which might be associated with the transformation or growth potential.

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INTRODUCTION

Estrogen is an important factor in supporting the growth, development, and differentiation of uterine endometrium from clinical evidences. Among estrogen effects on the endometrium, the growth of endometrium is critically related to estrogen status. Estrogen induces the expression of genes such as c-*fos* [1, 2], c-*jun* [3, 4], c-*myc* [5], c-*Ha-ras* [6, 7], and genes for EGF [8, 9], EGF receptor [10–12] and estrogen receptor in mammalian uterus. Oncoproteins among estrogen-inducible proteins in human uterine endometrial cancers might be, at least in part, related to the transformation activity and the growth of endometrial neoplasia.

To determine the contribution made by *ras* transcript to transformation or growth potential in endometrial cancers, we investigated the regulatory manner of one oncogene transcript, c-*Ha-ras* mRNA, under estrogen/progesterone predominant milieu in endometrial cancer cells, such as Ishikawa [13] and HHUA cells [14], which are estrogen-dependent for growth. Endometrial fibroblasts derived from uterine endometrium, as a substitute for normal endometrial stroma, were used as a control. Moreover, they were investigated with reference to activation and regulation of protein kinases (tyrosine kinase, protein kinase A, C and calmodulin kinase).

MATERIALS AND METHODS

Chemicals

Estradiol-17β, progesterone 8-(4-chlorophenylthio)-cAMP (CAMP), forskolin (FORS), cholera toxin (CT), 1-methyl-3-isobutyl-xanthine (MIX) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). One-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) was obtained from Seikagaku Corp. (Tokyo, Japan). Epidermal growth factor (EGF) was obtained from Austral Biologicals (San Ramon, CA, U.S.A.). Tyrphostin (TYR) was

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purchased from Gibco BRL. (Gaithersburg, MD, U.S.A.). All other reagents were of experimental grade.

Cell culture

Human endometria were obtained by endometrial biopsy from 6 patients (23–39 years old) with a regular menstrual cycle at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, from March 1993 to December 1993. Informed consent was obtained from all patients. The endometrium was washed with phosphate buffer solution containing amphotelicin B (1.5 μg/ml) and kanamycin (60 μg/ml), and minced with a scalpel. The minced tissue, in Eagle's MEM (Nissui Pharm., Tokyo, Japan) with 0.25% trypsin, was blended for 20 min and then pipetted to make a cell suspension. After addition of fetal bovine serum (FBS; Gibco BRL.) to the cell suspension to inactivate trypsin, the dispersed cells were recovered. The cells were cultured in MEM with amphotericin B and kanamycin overnight, and then the floating cells were discarded. After the 13th to 14th passage, spindle-shaped cells usually grew in a sheet-like manner, and occupied a whole square of the culture flask. Enriched collagen type I was detected in these spindle cells immunohistochemically, and thus they were considered to be fibroblasts from the endometrium.

Fibroblasts were cultured in 90% Eagle's MEM and 10% FBS. Afterwards, the culture was allowed to proceed in Eagle's MEM alone for 48 h, and then individual final concentrations of 10^{-12}–10^{-6} M estradiol, 10^{-12}–10^{-6} M progesterone, 0.5 mM CAMP, 3 μM FORS, 50 nM CT, 0.5 mM MIX, 1 μM TPA, 25 mM KCl, and 20 μM H-7 were settled in culture dishes.

Endometrial cancer cell lines of Ishikawa [13] and HHUA [14] were cultured in 90% RPMI 1640 (Nissui Pharm.) and 10% FBS, and in 85% Eagle's MEM and 15% FBS, respectively. The culture medium was changed to the same MEM without FBS 48 h before the addition of estrogen and progesterone to cells in culture dishes.

Reverse transcription–polymerase chain reaction (RT-PCR) to amplify c-Ha-ras transcripts

Total RNA was isolated from the cells by the acid guanidium thiocyanate–phenol–chloroform extraction method [15]. Total RNA (3 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units, Gibco BRL.) in 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin. 10 mM dithiothreitol (DTT), and 0.5 mM deoxynucleotides to generate cDNAs using random hexamers (50 ng, Gibco BRL.) at 37°C for 60 min. The RT reaction was heated at 94°C for 5 min to inactivate MMLV-RTase.

Ten cycles of PCR for c-*Ha-ras* mRNA alone [16], consisting of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1 min at 72°C for extension, were carried out with reverse transcribed cDNAs and 0.1 μM specific primers using IWAKI thermal sequencer TSR-300 (Iwaki Glass, Tokyo, Japan) with Vent DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.) in 10 mM KCl, 20 mM Tris–HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100 and 0.15 mM deoxynucleotides phosphates. Then an additional twenty cycles of PCR for c-*Ha-ras* mRNA and glyceraldehyde-3-phosphatedehydrogenase (GAPDH, a housekeeping gene) mRNA [17] as an internal standard were performed in the same manner as described before in the same tube. The following oligodeoxynucleotides were synthesized and used as a specific primer in PCR: sense for c-*Ha-ras*: 5′-ATGACGGAATATAAGCTGGT-3′, antisense for c-*Ha-ras*: 5′-CTGTACTGGTGGATGTCCTC-3′, sense for GAPDH: 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, and antisense for GAPDH: 5′-CATGTGGGCCATGAGGTCCACCAC-3′.

Analysis of quantities of c-Ha-ras expressions by Southern blot in PCR products

PCR products were applied to 1.2% agarose gel, and electrophoresis was performed at 50–100 V. PCR products were capillary-transferred to Immobilon transfer membrane (Millipore Corp., Bedford, MA, U.S.A.) for 16 h. The membrane was dried at 80°C for 30 min, and was UV-irradiated to fix PCR products tightly. PCR products on the membrane were prehybridized in 1 M NaCl, 50 mM Tris–HCl, pH 7.6, 1% SDS, at 42°C for 1 h, and hybridized in the same solution with the biotinylated oligodeoxynucleotides probes synthesized from the sequences of c-*Ha-ras* between the specific individual primers at 65°C overnight. Specific bands hybridized with the biotinylated probes were detected with Plex Luminescent Kits (Millipore Corp., Bedford, MA, U.S.A.), and the membranes were exposed X-ray films at room temperature for 10 min. The quantification of Southern blot was carried out with Bio Image (Millipore Corp., Ann Arbor, MI, U.S.A.). The intensity of specific bands was normalized with that of GAPDH mRNA.

Tyrosine kinase (TK) activity assay

All steps were carried out at 4°C, except where indicated. The cells (10–20 mg) were homogenized in HG buffer (5 mM Tris–HCl, pH 7.4, 5 mM NaCl, 1 mM $CaCl_2$, 2 mM ethyleneglycol-bis-[β-aminoethyl ether]-*N,N,N′,N′*-tetraacetic acid, 1 mM $MgCl_2$, 2 mM DTT, 25 μg/ml aprotinin, 25 μg/ml leupeptin) by a Polytron homogenizer (Kinematics, Luzern, Switzerland). This suspension was centrifuged in a microfuge at 12,000 rpm for 3 min to remove the nuclear pellet. The protein concentration of samples

was measured by the method of Bradford [18] to standardize TK activity. TK activity in samples was measured with 10 μCi/sample of [γ-^{32}P]ATP (3000–6000 Ci/mmol) by the Protein Tyrosine Kinase Assay System (Gibco BRL.).

RESULTS

Dose–response curve of estradiol for effects on c-Ha-ras expression in endometrial fibroblasts and cancer cells

The expression of c-*Ha-ras* mRNA in endometrial fibroblasts and endometrial cancer cells were analyzed by RT-PCR–Southern blot (RT-PCR-SB) [Fig. 1(A)]. The level of c-*Ha-ras* mRNA was standardized with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, and designated as an arbitrary unit (AU)/GAPDH mRNA.

The level of c-*Ha-ras* mRNA in endometrial fibroblasts and Ishikawa cells significantly ($P < 0.05$) increased by treatment with up to 10^{-8} M estradiol for 12 h, but not in HHUA cells [Fig. 1(B)]. Therefore, the concentration of estradiol required to achieve optimum c-*Ha-ras* expression appeared to be 10^{-8} M [Fig. 1(B)].

Dose–response curve of progesterone for effects on estradiol-induced c-Ha-ras expression in endometrial fibroblasts and cancer cells

The expression of c-*Ha-ras* mRNA was analyzed by RT-PCR–SB [Fig. 2(A)]. The estradiol-induced expression of c-*Ha-ras* mRNA in endometrial fibroblasts was significantly ($P < 0.05$) diminished by treatment with up to 10^{-6} M progesterone for 12 h, but not in Ishikawa nor in HHUA cells [Fig. 2(B)]. Therefore, the concentration of progesterone required to achieve maximum inhibition of c-*Ha-ras* expression appeared to be 10^{-6} M, based on progesterone solubility in the culture medium.

Time-course for effects of estradiol and/or progesterone on c-Ha-ras expression in endometrial fibroblasts and cancer cells

The expression of c-*Ha-ras* mRNA was analyzed by RT-PCR–SB [Fig. 3(A)]. The level of c-*Ha-ras* mRNA in endometrial fibroblasts and Ishikawa cells was significantly ($P < 0.05$) increased by treatment with estradiol, and reached a plateau in 12 h, but not in HHUA cells [Fig. 3(B)]. The estradiol-induced expression of c-*Ha-ras* mRNA was significantly

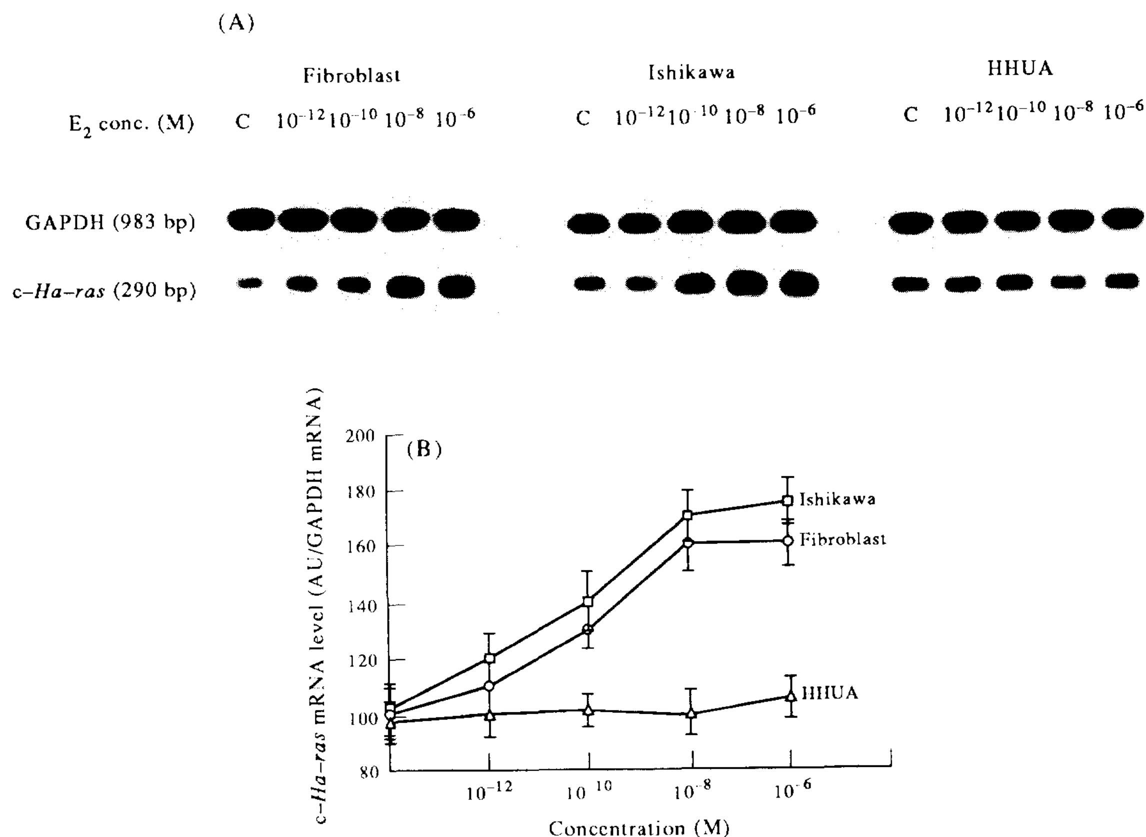


Fig. 1. Dose–response curve showing the effects of estradiol on c-*Ha-ras* expression in endometrial fibroblasts and cancer cells. Endometrial fibroblasts, Ishikawa and HHUA cells were incubated with 10^{-12}–10^{-6} M estradiol in Eagle's MEM medium for 12 h. Total RNA was isolated from them, and RT-PCR-SBA was carried out (A). The level of c-*Ha-ras* mRNA was standardized with that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA, and designated as an arbitrary unit (AU)/GAPDH mRNA. Data are the mean ± SD of 6 determinations (B). E_2, estradiol-17β.

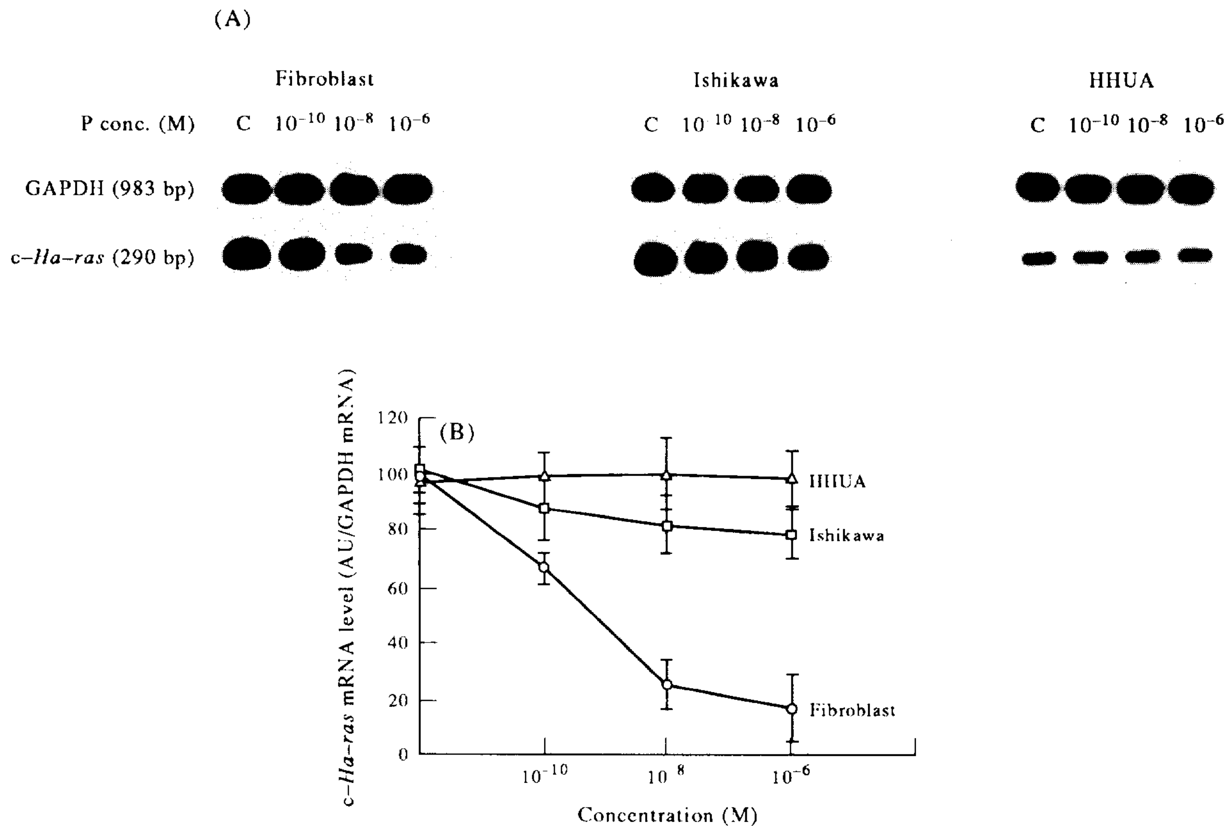


Fig. 2. Dose–response curve showing the effects of progesterone on estradiol-induced c-*Ha-ras* expression in endometrial fibroblasts and cancer cells. All cells were incubated with 10^{-8} M estradiol and/or 10^{-10}–10^{-6} M progesterone in Eagle's MEM medium for 12 h. RT–PCR–SBA was carried out (A). The level of c-*Ha-ras* mRNA was determined as described for Fig. 1. Data are the mean ± SD of 6 determinations (B). P, progesterone.

($P < 0.05$) diminished by progesterone in the fibroblasts, but not in Ishikawa nor HHUA cells [Fig. 3(B)].

Effects of estradiol, EGF and TYR on c-Ha-ras expression in endometrial fibroblasts and cancer cells

The expression of c-*Ha-ras* mRNA was analyzed by RT–PCR–SB [Fig. 4(A)]. The level of c-*Ha-ras* expression in endometrial fibroblasts and Ishikawa cells significantly ($P < 0.05$) increased for 12 h with treatment with EGF and/or estradiol, but not in HHUA cells [Fig. 4(B)]. The combination of EGF and estradiol exerted no additive or synergistic effect on c-*Ha-ras* expression in the former two cells [Fig. 4(B)]. 1 h pretreatment with TYR significantly ($P < 0.05$) diminished the increase induced by either estradiol or EGF in the fibroblasts and Ishikawa cells, but not in HHUA cells [Fig. 4(B)].

Effects of estradiol, CAMP, FORS, CT, MIX, TPA and KCl on c-Ha-ras expression in endometrial fibroblasts and cancer cells

The expression of c-*Ha-ras* mRNA was analyzed by RT–PCR–SB [Fig. 5(A)]. The level of c-*Ha-ras* expression was not affected by CAMP, FORS, CT, MIX, TPA and KCl, except for estradiol, in all three types of cells [Fig. 5(B)].

Effects of estradiol and/or progesterone on TK activity in endometrial fibroblasts and cancer cells

TK activity was significantly ($P < 0.05$) increased in endometrial fibroblasts and Ishikawa cells by treatment with estradiol for 2 h, and persisted to some extent, but transiently in HHUA cells for 6 h (Fig. 6). The estradiol-induced TK activity was significantly ($P < 0.05$) diminished by progesterone in fibroblasts and HHUA cells, but not in Ishikawa cells (Fig. 6).

DISCUSSION

Estrogen induces c-*Ha-ras* expression in rodent uterus [6, 7]. In human uterine endometrium, the expression of c-*Ha-ras* is higher at the proliferative phase than at the secretory phase of the menstrual cycle [19]. Treatment with estradiol dipropionate significantly elevated the expression of c-*Ha-ras* mRNA in the endometrium of the human subject [19]. Endometrial fibroblasts derived from uterine endometrium as a substitute for normal endometrial stroma were used as controls and endometrial cancer cells were used to analyze the induction manner of c-*Ha-ras* transcripts in endometrial cancers, some of which are estrogen-dependent in growth. The expression of c-*Ha-ras* in the fibroblasts was significantly increased by

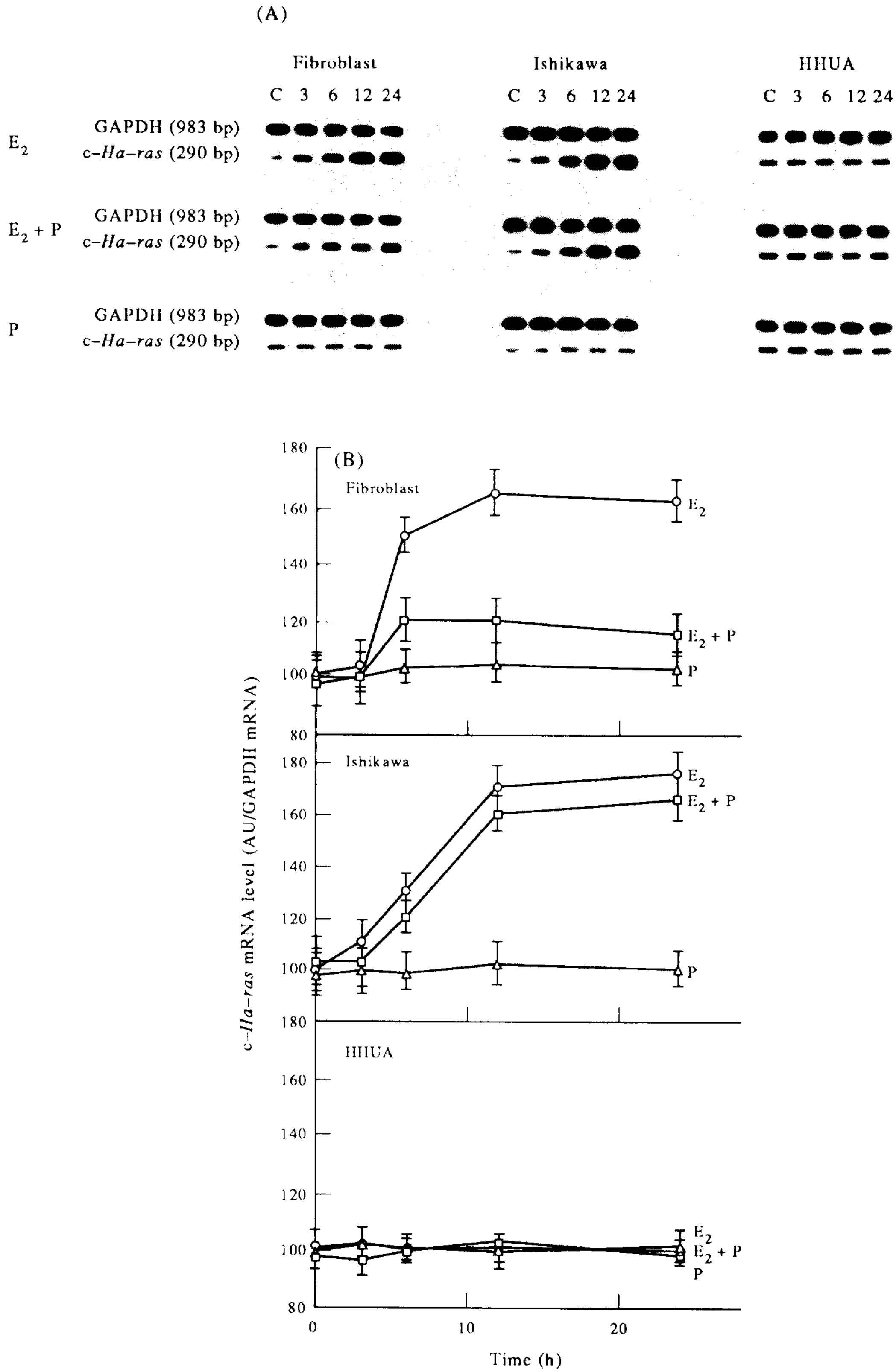
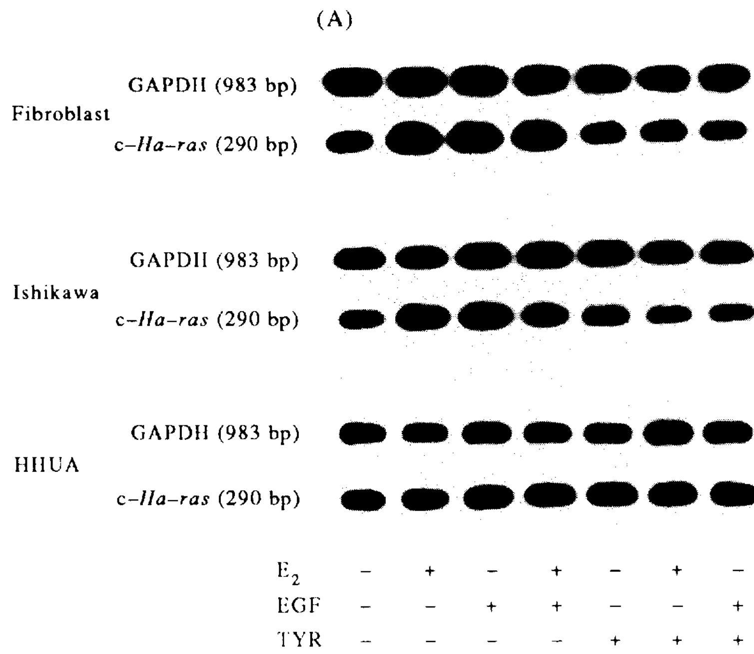


Fig. 3. Time-course for effects of estradiol and/or progesterone on c-*Ha-ras* expression in endometrial fibroblasts and cancer cells. All cells were incubated with 10^{-8} M estradiol and/or 10^{-6} M progesterone in Eagle's MEM medium for various periods of time. RT-PCR-SBA was carried out (A). The level of c-*Ha-ras* mRNA was determined as described for Fig. 1. Data are the mean ± SD of 6 determinations (B).



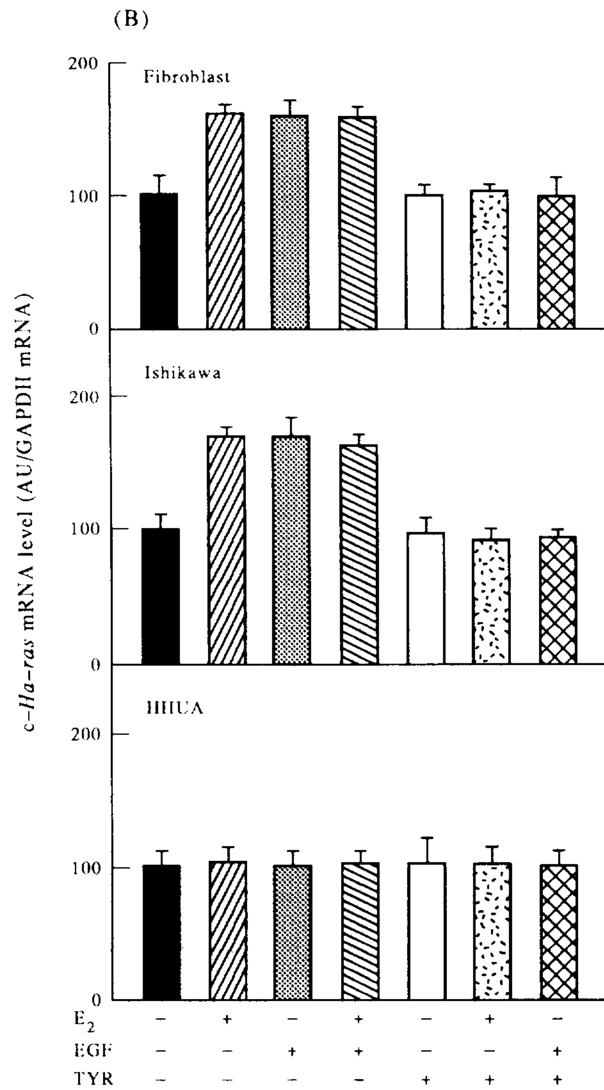


Fig. 4. Effects of estradiol, EGF and TYR of c-*Ha-ras* expression in endometrial fibroblasts and cancer cells. All cells were incubated with 10^{-8} M estradiol, 1 ng/ml EGF, and 15 μM TYR in Eagle's MEM medium for 12 h. RT–PCR–SBA was carried out (A). The level of c-*Ha-ras* mRNA was determined as described for Fig. 1. Data are the mean $\pm$ SD of 6 determinations (B). EGF, epidermal growth factor; TYR, tyrphostin.

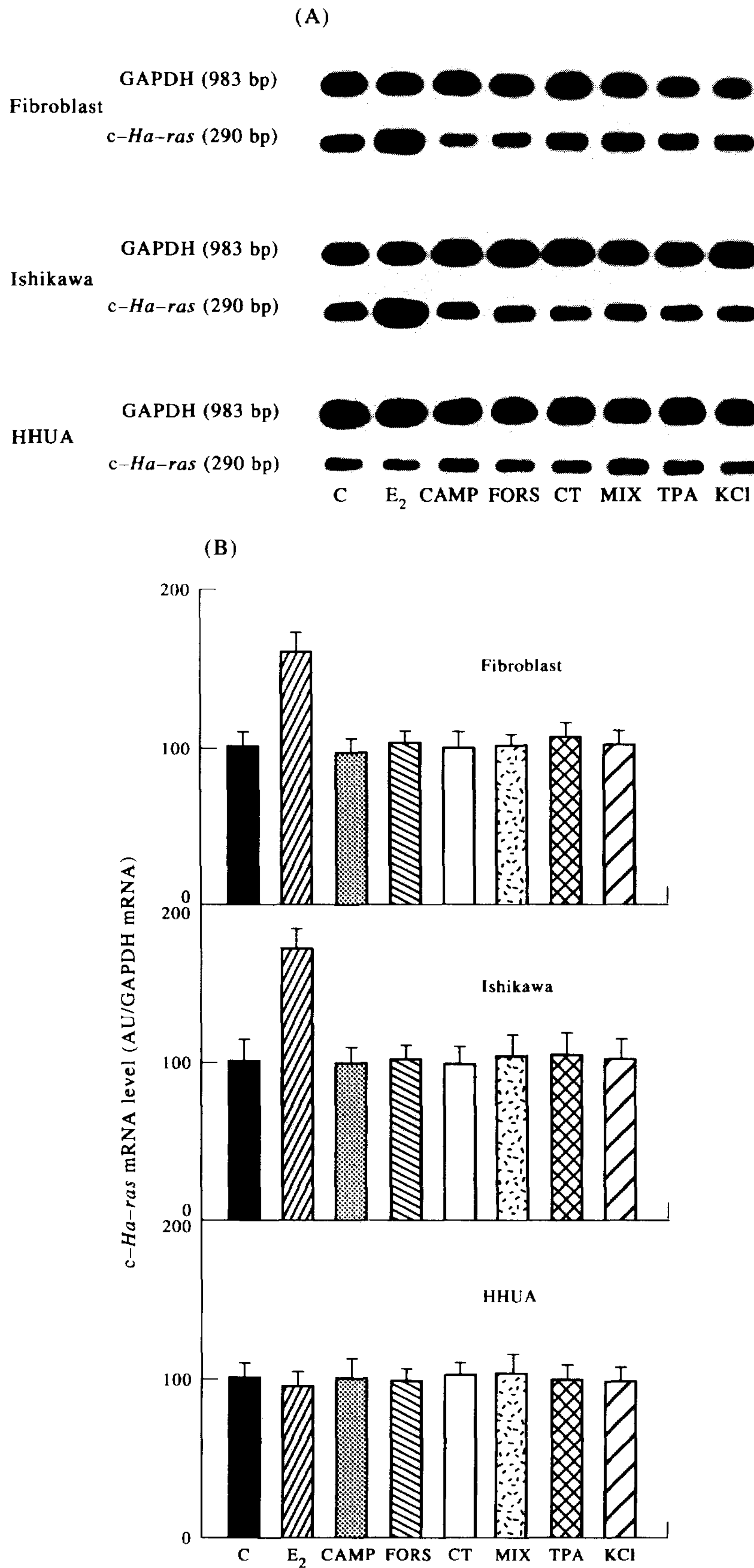


Fig. 5. Effects of estradiol, CAMP, FORS, CT, MIX, TPA, and KCl on c-*Ha-ras* expression of human endometrial fibroblasts and cancer cells. All cells were incubated with 10^{-8} M estradiol, 0.5 mM CAMP, 3 μM FORS, 50 nM CT, 0.5 mM MIX, 1 μM TPA, and 25 mM KCl in Eagle's MEM medium for 12 h. RT-PCR-SBA was carried out (A). The level of c-*Ha-ras* mRNA was determined as described for Fig. 1. Data are the mean $\pm$ SD of 6 determinations (B). CAMP, 8-(4-chlorophenyl-thio)-cAMP; FORS, forskolin; CT, cholera toxin; MIX, 1-methyl-3-isobutyl-xanthine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

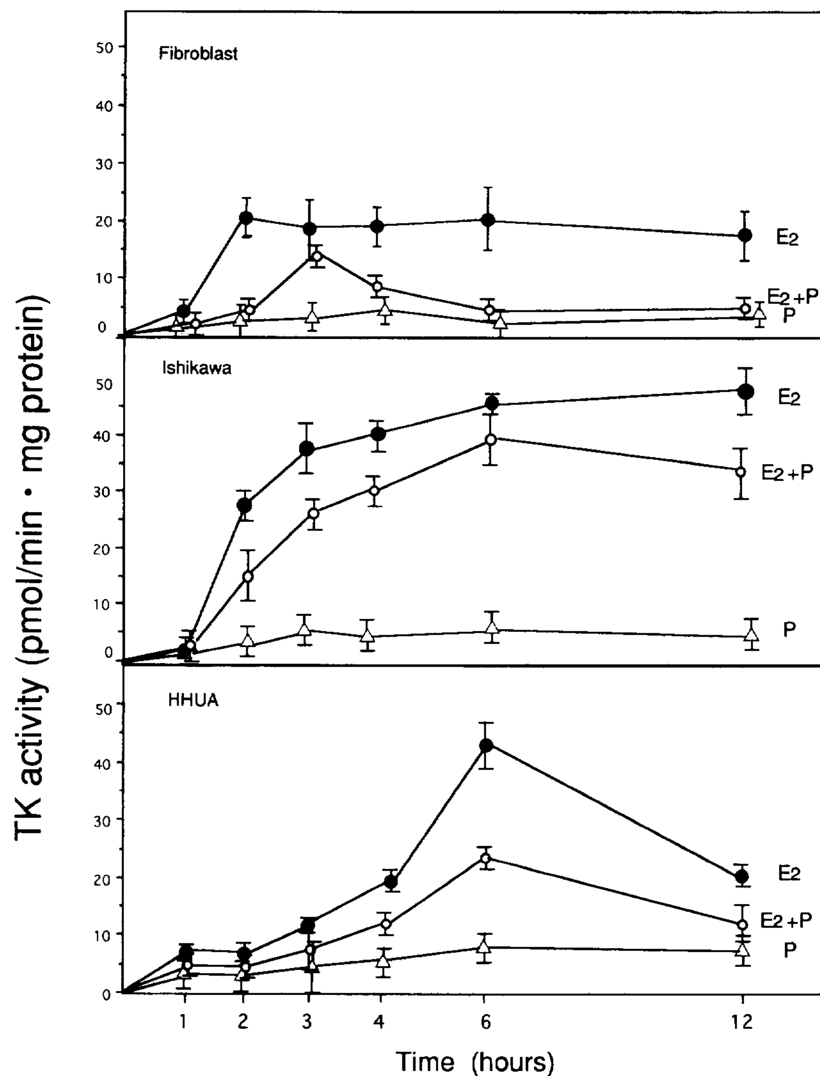


Fig. 6. Time-course for effects of estradiol and/or progesterone on TK activity of human endometrial fibroblasts and cancer cells. All cells were incubated with 10^{-8} M estradiol and/or 10^{-6} M progesterone in Eagle's MEM medium for various periods of time. TK activity in the cells was measured with 10 μCi/sample of [γ-^{32}P]ATP by the Tyrosine Kinase Assay System Kit (Gibco BRL. Gaithersburg, MD, U.S.A.). Data are the mean ± SD of 6 determinations.

estradiol, while progesterone significantly diminished it. On the other hand, in Ishikawa cells, which are estrogen-dependent in growth, the expression induced by estrogen was not significantly diminished by progesterone. In the control, c-*Ha-ras* expression is regulated positively by estrogen, and negatively by progesterone in the physiological state.

c-*Ha-ras* gene encodes *ras* p21 (RAS) among low molecular guanosine-5′-triphosphate (GTP) binding proteins [20, 21]. The exchange of guanosine-5′-diphosphate (GDP) to GTP in the complex with RAS by exogenous stimulants can be evaluated as a biological activity of RAS. The functionally activated RAS is related to cellular growth, differentiation, and transformation as follows. EGF and platelet derived growth factor (PDGF) increase the ratio of RAS–GTP to RAS–GDP in proliferative Swiss 3T3 cells [22, 23]. Macrophage-colony stimulating factor (M-CSF) and insulin increase the ratio of RAS–GTP to RAS–GDP, overexpressing their corresponding receptor in those cells with growth [23, 24]. In NIH3T3 cells transformed with *src*, *abl* and *erbB-2*, the ratio of RAS–GTP to RAS–GDP increases without any stimulant [23, 25]. Therefore, RAS activated by TK, such as receptor-type TK (*erbB-2* coding protein, and the receptors for EGF, PDGF, M-CSF and insulin), and non-receptor-type TK (coding proteins of *src* and *abl*), is considered to be a transducer for cellular growth. The persistent activation of RAS might contribute, in part, to cellular transformation. In addition, PKC in T cells activated by lectin or anti-CD antibody seems to inhibit GTPase-activating-protein activity, resulting in enhanced ratios of RAS–GTP to RAS–GDP [26].

TK activity in endometrial fibroblasts was increased by estradiol, and this activity was diminished by progesterone. In Ishikawa cells, the activity was increased

by estrogen, the increase of which was not significantly diminished by progesterone. Progesterone regulation might be lost in Ishikawa cells. In HHUA cells, TK activity was increased transiently by estradiol, however, exerting no effect on induction of c-*Ha-ras* expression. Therefore, persistent activation of TK is needed to induce c-*Ha-ras* expression. In endometrial fibroblasts and Ishikawa cells, EGF, an activator of TK, increased c-*Ha-ras* expression as did estradiol. Pretreatment with TYR, an inhibitor of TK, inhibited the estrogen-inducible overexpression of c-*Ha-ras*. The combination of both estradiol and EGF at maximum effective concentration exerted no additive or synergistic effect on induction of c-*Ha-ras* expression. Therefore, estrogen leads to persistent overexpression of c-*Ha-ras* via TK activation in transformed cells.

On the other hand, protein kinase A (PKA) activators (CAMP, FORS, CT, and MIX), a PKC activator (TPA), and a calmodulin kinase activator (KCl) did not affect c-*Ha-ras* expression in endometrial fibroblasts, Ishikawa cells and HHUA cells. Therefore, PKA, PKC and calmodulin kinase do not link directly to c-*Ha-ras* expression in these cells.

In conclusion, persistent activation of TK leads to overexpression of c-*Ha-ras* under estrogen predominant milieu in some endometrial cancers, in which progestational regulation might be lost. These might be associated with the maintenance of transforming activity.

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