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Inhibition of epidermal growth factor receptor functions by tyrosine kinase inhibitors in NIH3T3 cells

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Epidermal growth factor (EGF) induces transformed phenotypes in EGF receptor-overexpressing NIH3T3 (ER12) cells. Tyrosine kinase inhibitors such as erbstatin and its stable analogue methyl 2,5-dihydroxycinnamate inhibited the EGF-induced phenotypic changes in these cells; while 5′-*O*-methylerbstatin, an inactive analogue, did not. Methyl 2,5-dihydroxycinnamate inhibited intracellular tyrosine kinase activity in EGF-treated ER12 cells. Methyl 2,5-dihydroxycinnamate also delayed the EGF-induced DNA synthesis from the quiescent phase ER12 cells without showing irreversible cytotoxicity. It inhibited the DNA synthesis most efficiently at the early G_1 phase. Thus, tyrosine kinase inhibitors may modify malignant phenotypes in EGF receptor-overexpressing neoplasms.

Epidermal growth factor; Tyrosine kinase; Erbstatin; Cytoskeleton; Fibronectin; DNA synthesis

1. INTRODUCTION

Overexpression of epidermal growth factor (EGF) receptors has been reported in various tumour cell lines, and it has been suggested that amplified normal EGF receptors could contribute to tumorigenesis [1–3]. The ER12 cell line is a transfectant of human EGF receptor cDNA to the NIH3T3 cell line and shows various types of phenotypic transformation in response to EGF [4]. Including the ER12 cell line, several laboratories established EGF receptor-transfected cell lines and reported ligand-dependent transformation of these cells [5,6]. For instance, in the presence of EGF, the cells become less dependent on serum, lose contact inhibition, and acquire growth advantage and anchorage-independent growth ability.

The activated EGF receptor has tyrosine kinase activity in its cytoplasmic portion, which is essential for the EGF-EGF receptor-mediated proliferation signal. Many oncogene products such as Erb B, Erb B2, Fms, Src, and Abl also have tyrosine kinase activity. Although the main target of the tyrosine kinase is not known, the kinase of the EGF receptor is considered to act on phospholipase C-γ [7], phosphatidylinositol 3′-kinase [8], and GAP [9].

Erbstatin was isolated from *Streptomyces* as an inhibitor of EGF receptor tyrosine kinase, and methyl 2,5-dihydroxycinnamate (2,5-MeC) is a stable analogue of it [10]. Both were shown to delay the onset of EGF-induced DNA synthesis in normal rat kidney (NRK) cells [11]. They also inhibited Src-induced disruption of actin stress fiber organization and enhanced fibronectin expression in RSV^{ts}-NRK cells [12]. In the present study, we examined the effect of 2,5-MeC on EGF-induced morphological transformation, cytoskeletal organization, fibronectin expression, and DNA synthesis in the ER12 cell line.

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2. MATERIALS AND METHODS

2.1. *Materials*

The ER12 cell line was prepared as described previously [4]. Calf serum (CS) was purchased from Gibco. Epidermal growth factor was purchased from Biomedical Technologies. Erbstatin was isolated from *Streptomyces* as described [10]. Methyl 2,5-dihydroxycinnamate and 5′-*O*-methylerbstatin were synthesized in our laboratory. Anti-mouse fibronectin polyclonal antibody was purchased from UCB Bioproducts; and rhodamine-conjugated phalloidin, from Molecular Probes Inc. ^{125}I-labelled protein A (3.7 MBq/ml) was purchased from Amersham.

2.2. *Cell culture*

ER12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% CS at 37°C in a 5% CO_2/95% air atmosphere.

2.3. *Cellular morphology*

ER12 cells (5×10^4) plated in 24-mm plastic dishes were grown for 16 h before use. Then 10 ng/ml of EGF and test chemicals were added and the cells were examined by phase-contrast microscopy. Appropriate controls were run to test the effects of the solvents. All the morphological and fluorescence staining experiments were repeated twice or more.

2.4. *Fluorescence staining*

ER12 cells grown on cover glasses were treated with 10 ng/ml of

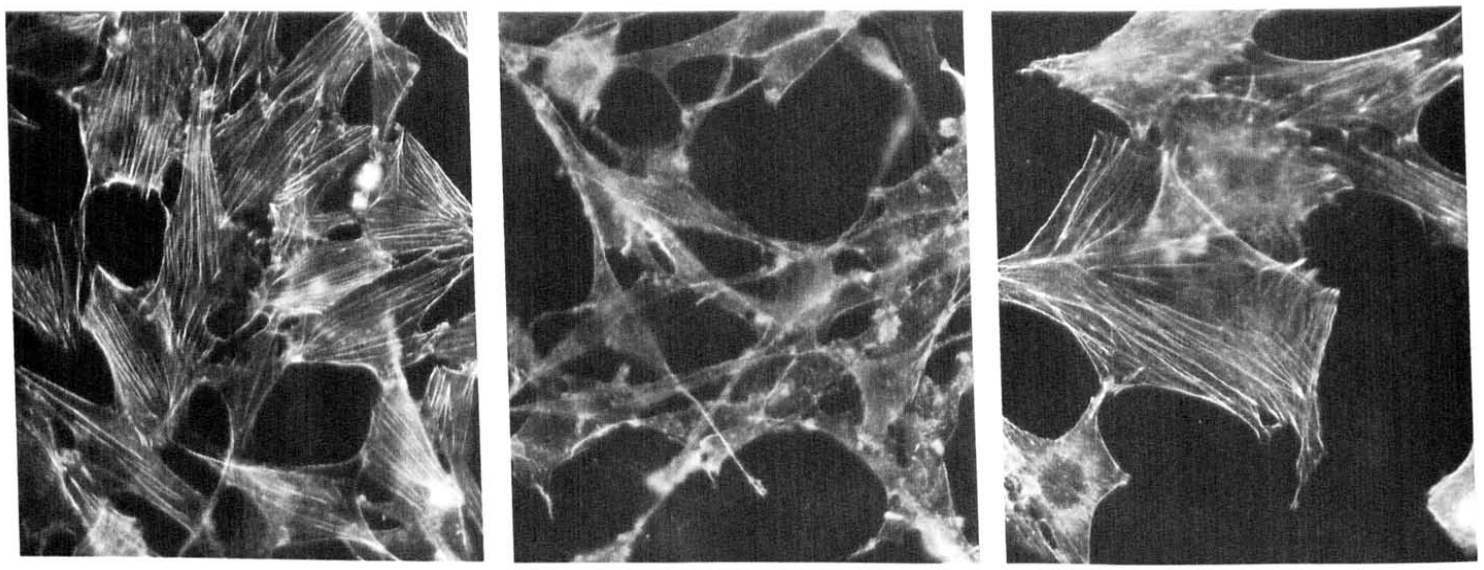

Fig. 1. Inhibition of EGF-induced disruption of actin fiber organization by 2,5-MeC in ER12 cells. The cells were incubated alone (A) with 10 ng/ml of EGF (B), or with EGF and 1.6 μg/ml of 2,5-MeC (C) for 16 h, and then stained with rhodamin-phalloidin for visualization of actin.

EGF and test chemicals for 16 h and fixed with 3.5% paraformaldehyde in Ca^{2+},Mg^{2+}-free phosphate-buffered saline (PBS) for 20 min at room temperature. After a brief rinse in Ca^{2+},Mg^{2+}-free PBS, the fixed cells were permeabilized with 0.5% Triton X-100 in Ca^{2+},Mg^{2+}-free PBS. The cover glasses were washed with Ca^{2+},Mg^{2+}-free PBS and incubated for 20 min at 37°C with rhodamine-conjugated phalloidin to detect polymerized actin or with the specific antibody to detect fibronectin. They were washed with Ca^{2+},Mg^{2+}-free PBS and examined by fluorescence microscopy.

2.5. *Immunoblotting*

ER12 cells (1.5×10^5) plated in 35-mm plastic dishes were grown for 16 h before use. The cells were treated with 10 ng/ml of EGF and test chemicals for 16 h. After a brief rinse with Ca^{2+},Mg^{2+}-free PBS, the cells were extracted with 200 μl of radioimmuno precipitation (RIPA) buffer for 30 min at 4°C. The cell lysates were centrifuged at 15,000 × *g* for 5 min. Then 100 μl of the supernatant was added to 100 μl of sample buffer (42 mM Tris-HCl, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol, 0.002% bromophenol blue, pH 6.3) and electrophoresed on a 7% polyacrylamide gel. Proteins were transferred to a nitrocellulose filter and incubated with anti-fibronectin antibody for 30 min at 37°C. After a brief rinse, the filter was reacted with ^{125}I-protein A for 16 h at 4°C. Fibronectin bands were visualized by autoradiography.

2.6. *EGF receptor tyrosine kinase activity*

When the immunoblotting analysis was performed with anti-phosphotyrosine antibody, addition of 2,5-MeC or erbstatin gave unwanted backgrounds. Therefore, the cell lysate was precipitated with the anti-EGF receptor antibody first, and then the in vitro tyrosine kinase of the precipitate was assayed. Intact ER12 cells (about 4×10^5) were treated with various concentrations of 2,5-MeC in serum-free DMEM for 15 min, and EGF (100 ng/ml) was added for further 5 min. The cells were collected and solubilized with 1% Triton X-100 in 20 mM HEPES (pH 7.4) containing 10% glycerol, 0.1 mg/ml of leupeptin, 1 mM PMSF, and 100 μM Na_3VO_4 at 4°C for 30 min. An aliquot of solubilized material was reacted with anti-EGF receptor antibody for 16 h at 4°C, and the complexes formed were immunoprecipitated with anti-mouse IgG and collected with Sepharose-linked protein A. The immune complexes were suspended in 20 mM HEPES buffer (pH 7.4) consisting of 1 mM $MnCl_2$, 10 mM $MgCl_2$, 100 μM Na_3VO_4, and reacted with [γ-^{32}P]ATP and 4 μg of enolase in 50 μl for 15 min at 30°C. The reaction was terminated by addition of the same

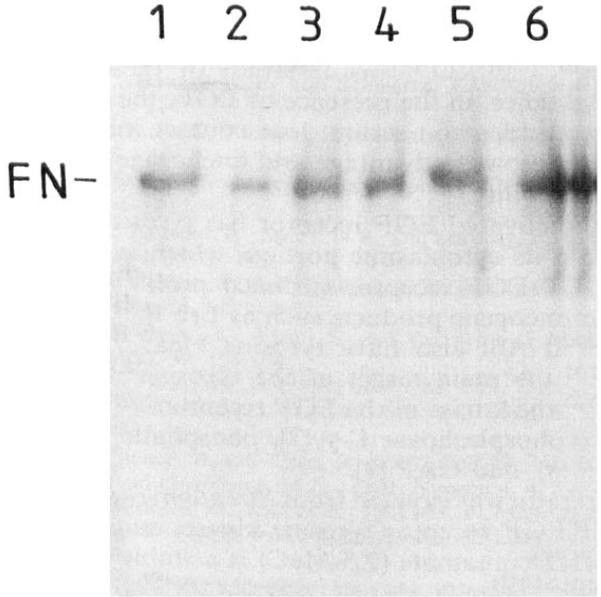

Fig. 2. Inhibition of EGF-induced reduction in fibronectin expression by tyrosine kinase inhibitors in ER12 cells. The cells were incubated without additive (Lane 1), or with 10 ng/ml of EGF (2), EGF and 3.2 (3) or 1.6 (4) μg/ml of erbstatin, or EGF and 3.2 (5) or 1.6 (6) μg/ml of 2,5-MeC, for 16 h, and then processed for immunoblotting as described in Section 2.

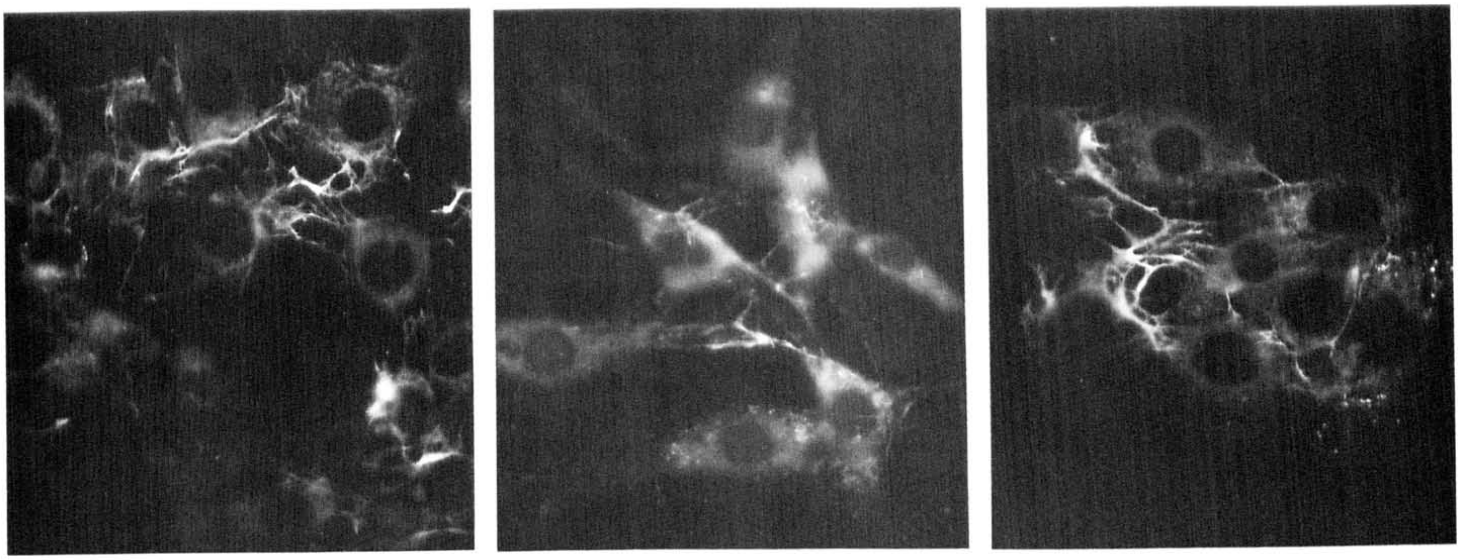

Fig. 3. Inhibition of EGF-induced fibronectin network disappearance by 2,5-MeC in ER12 cells. The cells were treated alone (A), with 10 ng/ml of EGF (B), or with EGF and 1.6 μg/ml of 2,5-MeC (C) for 16 h, and then stained with anti-fibronectin antibody to detect fibronectin.

volume of sample buffer and the mixture boiled for 10 min. The sample was finally electrophoresed on a 10% polyacrylamide gel and autoradiographed.

3. RESULTS

Methyl 2,5-dihydroxycinnamate inhibited the growth of ER12 cells with an IC_{50} of 0.5 μg/ml. In the presence of EGF, it inhibited the EGF-stimulated growth with almost the same IC_{50} value. Erbstatin inhibited the cell growth with an IC_{50} value of 1.65 μg/ml both in the absence and in the presence of EGF.

ER12 cells show normal fibroblastic morphology in the absence of EGF, but within 16 h of the addition of 10 ng/ml EGF the cells assume a thin and slightly criss-crossing transformed morphology [4]. Methyl 2,5-dihydroxycinnamate and erbstatin did not clearly inhibit the EGF-induced morphological change because the effect of EGF was not prominent. However, as shown in Fig. 1A,B, actin stress fiber organization was clearly observed in non-treated ER12 cells but not in EGF-treated cells. When EGF was added in the presence of 0.8–1.6 μg/ml of 2,5-MeC, stress fiber organization was retained at the control level (Fig. 1C). The effect of 2,5-

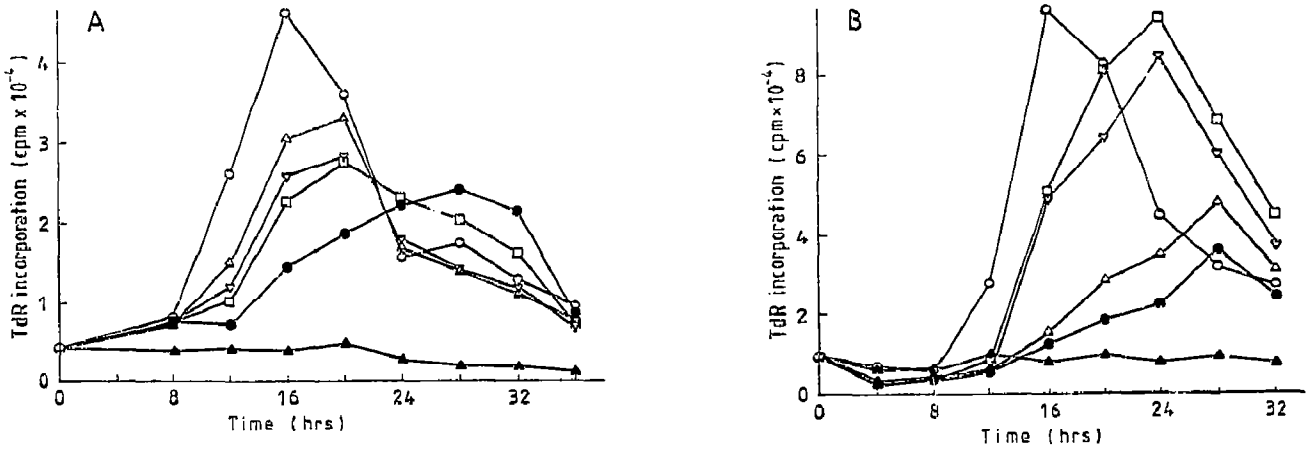

Fig. 4. Inhibition of EGF-induced DNA synthesis in quiescent ER12 cells by 2,5-MeC. (A) Quiescent ER12 cells were untreated (▲), or treated with 40 ng/ml of EGF alone (○), or EGF and 1 (△), 2 (▽), 4 (□), or 8 (●) μg/ml of 2,5-MeC. Labelled thymidine was added to the cells for 1 h at the indicated time. (B) The cells were untreated (▲), or treated with EGF alone (○), or EGF and 8 μg/ml of 2,5-MeC at 0–4 h (△), 4–8 h (▽), 8–12 h (□), or 0–32 h (●) from the time of EGF addition.

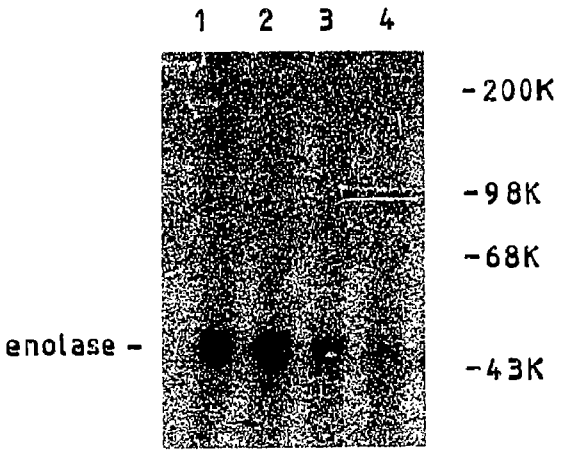

Fig. 5. In situ inhibition of EGF receptor-associated tyrosine kinase by 2,5-MeC. The cells were untreated (Lane 1), or treated with 100 ng/ml of EGF (2), or EGF and 3 (3) or 6 (4) μg/ml of 2,5-MeC for 15 min. Precipitation of the cell lysate and the in vitro tyrosine kinase assay were carried out as described in Section 2.

MeC or erbstatin was reversible, since after the removal of the chemicals the cellular cytoskeletal change was induced by addition of EGF. An inactive analogue 5′-*O*-methylerbstatin did not inhibit the cytoskeletal change induced by EGF.

Fibronectin gene expression is usually less in transformed cells than in normal cells. As shown in Fig. 2, the amount of fibronectin decreased 16 h after addition of EGF, but 2,5-MeC or erbstatin inhibited this decrease. These inhibitory effects were confirmed by immunofluorescence staining (Fig. 3).

Methyl 2,5-dihydroxycinnamate delayed the onset of EGF-induced DNA synthesis in quiescent ER12 cells at 1.0–8.0 μg/ml, as shown in Fig. 4A, and it inhibited the DNA synthesis most efficiently when added at the early G_1 phase (Fig. 4B).

Methyl 2,5-dihydroxycinnamate was confirmed to inhibit EGF receptor-associated tyrosine kinase in situ, as shown in Fig. 5. The amount of EGF receptor was not affected by 2,5-MeC (data not shown).

4. DISCUSSION

The ER12 cells showed phenotypic transformation in response to EGF, including morphological change, growth advantage, loss of stress fiber organization, and decrease in fibronectin expression; and all of these phenotypic changes were inhibited by erbstatin or 2,5-MeC, but not by its inactive analogue 5′-*O*-methylerbstatin. The tyrosine kinase inhibitors reversibly inhibited the EGF-induced DNA synthesis. Pulse addition of the inhibitor indicated that tyrosine kinase activity is essential in G_0/G_1 phase.

Loss of stress fibers and a decrease in fibronectin expression are often observed in malignant transformation. Because tyrosine kinase inhibitors inhibited EGF-induced cytoskeletal changes and decrease in fibronectin expression, tyrosine kinase may regulate cytoskeletal organization and adhesion of the cells directly or indirectly. Previously it was reported that EGF receptors are associated with cytoskeletal structure [13]. Phosphatidylinositol turnover inhibitors such as inostamycin and psi-tectorigenin inhibited EGF-induced cytoskeletal change in A431 cells [14], but not obviously in ER12 cells. Therefore, it might be less likely that tyrosine kinase regulates the cytoskeleton through phosphatidylinositol turnover.

EGF receptors on the tumour cell surface would contribute to the development of neoplasms in vivo in response to EGF and related growth factors. It may be possible to inhibit such development by use of tyrosine kinase inhibitors of low molecular weight.

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