

Perturbation of EGF-induced MAP kinase activation by TGF- β 1

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Abstract

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TGF- β 1 regulates both cellular growth and phenotypic plasticity important for maintaining a growth advantage and increased invasiveness in progressively malignant cells. Recent studies indicate that TGF- β -1 stimulates the conversion of epithelioid to fibroblastoid phenotype which presumably leads to the inactivation of growth-inhibitory effects by TGF- β 1 (Portella et al. (1998) *Cell Growth and Differentiation*, 9: 393-404). Therefore, the investigation of TGF- β 1 signaling that leads to altered growth and migration may provide novel targets for the prevention of increased cell growth and invasion. Although much attention has been paid to TGF- β 1 responses in epithelial cells, the above studies suggest that examination of signal transduction pathways in fibroblasts are important as well. Data from our laboratory are consistent with the concept that TGF- β 1 can act as a regulatory switch in density-dependent C3H 10T1/2 fibroblasts capable of either promoting or delaying G1 traverse. The regulation of this switch is proposed to occur prior to pRb phosphorylation, namely prior to activation of cyclin-dependent kinases. The current study is concerned with the evaluation of a key cyclin (cyclin D1) which activates cdk4 and p27^{KIP1} which in turn inhibit cdk2 in the proliferative responses of epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) and their modulation by TGF- β 1. Although the molecular events that lead to elevation of cyclin D1 are not completely understood, it appears likely that activation of p42/p44^{MAPK} kinases is involved in its transcriptional regulation. TGF- β 1 delayed EGF- or PDGF-induced cyclin D1 expression and blocked the induction of active p42/p44^{MAPK}. The mechanism by which TGF- β 1 induces a block in p42/p44^{MAPK} activation is being examined and the possibility that TGF- β 1 regulates phosphatase activity is being tested.

Key words

- Transforming growth factor
- MAP kinase
- Cyclin D1

Although there is much support for a negative regulatory role of TGF- β in tumorigenesis, it is our view that TGF- β 1 not only provides a growth advantage in several types of progressively malignant epithelial cells but permits the development of a more invasive phenotype. This stems largely from observations by Balmain and colleagues and by

others (cf. 1) that I) overexpression of TGF- β -RII leads to an increased incidence of the highly invasive mouse skin spindle carcinoma (2) and II) expression of a dominant-negative TGF β -type II receptor in squamous cell clones prevents spontaneous conversion to the fibroblastoid type after subcutaneous injection in nude mice (1). These observa-

tions support the idea that TGF- β 1 can act directly on keratinocytes *in vivo* to induce the reversible epithelial-mesenchymal conversion of a malignant metastatic keratinocyte cell line. There is also evidence that the responses to TGF- β 1 are markedly altered when well-differentiated tumor cells become progressively malignant, i.e., colon carcinoma cells switch their response to TGF- β 1 with tumor progression (3). In other words, metastatic colon carcinoma cells in primary culture respond to TGF- β 1 by proliferation, whereas the growth of moderate to well-differentiated primary site colon carcinomas is inhibited by TGF- β 1 (4). Other recent studies have shown an association of TGF- β 1 with increased malignancy in other tumor types (5). While TGF- β 1 inhibits normal mammary cell growth and morphogenesis *in vivo* (6), the increased abundance of TGF- β 1 protein in breast carcinoma is positively associated with disease progression as examined by immunocytochemistry using isotype-specific antisera (7,8). Also, the metastatic potential of mammary adenocarcinoma cells was increased by TGF- β 1 (9) whereas the non-metastatic tumor cells were subject to growth inhibition. In a series of rat bladder carcinoma cell lines which were clonally derived, the expression of TGF- β 1 mRNA increased in conjunction with the biological aggressiveness of the cell lines (10). Thus, in both human tumors and in experimental model systems, TGF- β 1 is associated with increased disease progression in epidermal, colon, breast, and bladder cancer. Studies by Friedman and colleagues (3) reported that the *in vivo* mixture of TGF- β 1 growth-inhibited and of TGF- β 1 growth-stimulated tumor cells found in the primary tumor were replicated using cell lines derived from the same tumor, and provide a basis for the growth advantage possessed by the more invasive cells.

Oft et al. (11) reported that normal and Ras-transformed mammary epithelial cells grown in collagen gels maintain their epithe-

lial characteristics. However, treatment with TGF- β 1 of normal cells results in growth arrest but the same treatment of Ras-transformed epithelial cells causes them to become fibroblastoid, invasive, and resistant to growth inhibition by TGF- β 1. Thus, metastasis of epithelial tumor cells can be associated with the acquisition of fibroblastoid features.

These studies suggest that the examination of signal transduction pathways in a mesenchymal cell line is of inherent value as is the study of negative effects of TGF- β 1. The study of TGF- β 1 in a cell line such as C3H 10T1/2 mouse embryonic fibroblasts is useful in that these cells provide ease of synchronization to obtain a relatively homogeneous cell population with which to follow cell cycle stimulation by TGF- β 1 and its interaction with signal transduction pathways initiated by other mitogens.

We and others have observed that TGF- β 1 can act as a regulatory switch by delaying epidermal growth factor (EGF) or platelet-derived growth factor (PDGF)-induced DNA synthesis (12,13). It was, therefore, of interest to examine the mechanism by which TGF- β 1 elicits these responses. The key factors involved in G1-S checkpoint control have recently been elucidated. That is, cell cycle progression is dependent on G1 cdk activity which is regulated by two counter-regulatory protein types, i.e., cyclins, the positive effectors and cdk inhibitors (cdkis), the negative effectors. Cyclin D1 and p27 are important representatives of each type, respectively. In C3H 10T1/2 cells, EGF stimulates cell growth by up-regulating cyclin D1 whereas TGF- β 1 stimulates DNA synthesis by down-regulating p27^{KIP1} (13,14). Despite the independent mechanisms by which each ligand stimulated DNA synthesis, when both ligands were present, there was a delay in the kinetics as observed when EGF was added alone. This led us to compare the levels of cyclin D1 and p27^{KIP1} under these conditions. We found that al-

though p27^{KIP1} was unchanged when both ligands were present, TGF- β 1 prevented the up-regulation of EGF-induced cyclin D1 levels (14). It was, therefore, of interest to examine the effects of TGF- β 1 on pathways contributing to EGF-induced cyclin D1 expression.

Evidence for the involvement of the MEK-MAPK (MAPK = mitogen-activated protein kinase; MEK = MAPK kinase) pathway in cyclin D1 expression has been presented (15). MAPKs augment the activity of several transcription factors including the activator protein-1 complex (AP-1, c-Fos/c-Jun), ATF-2, and members of the ETS transcription factor family. The donation of PD 098059, a specific inhibitor of the activation of mitogen-activated protein kinase *in vitro* and *in vivo*, by Dr. Alan R. Saltiel, Parke Davis Research Division, Warner Lambert Company, Ann Arbor, MI, has allowed us to carry out initial studies to examine its effect on EGF-induced DNA synthesis in C3H 10T1/2 cells as described below.

As seen in Figure 1, PD 098059 (50 μ M) inhibited EGF-induced DNA synthesis. In contrast, preliminary studies with PD 098059 (10 μ M) indicate that although a less pronounced inhibition of EGF-induced DNA synthesis was observed, there was no effect of PD 098059 on TGF- β 1 induced DNA synthesis. Thus, these findings are consistent with the possibility that MAP kinase is required for activation of cyclin D1 synthesis by EGF but that it may not be critical for TGF- β 1-induced DNA synthesis. We therefore carried out studies to determine its effect on EGF-induced MAPK activation.

With the use of an anti-active p42/p44^{MAPK} antibody from Promega Corp. (Madison, WI, USA), we examined the effect of TGF- β 1 on the activation of p42/p44^{MAPK}. EGF induces marked stimulation of p42/p44^{MAPK} activity within minutes and significant enhancement at 10 min post-treatment as does tetradecanoyl-phorbol acetate (TPA). It was also observed that TGF- β 1 significantly reduced

EGF-induced p42/p44^{MAPK} activation minutes after both factors were applied at the same time (Figure 2). In view of previous studies indicating a requirement of p42/p44^{MAPK} activation for cyclin D1 synthesis, our findings are consistent with the possibility that TGF- β 1 inhibits p42/p44^{MAPK} activity, which conceivably prevents EGF-induced cyclin D1 activity.

We also were able to evaluate the contribution of the MAPK pathway to the induction of cyclin D1 and p27^{KIP1} down-regulation with the use of a ligand, namely, the phorbol ester TPA, which induced proliferative responses by both mechanisms (16). It was observed in this study that with postconfluent, quiescent fibroblasts, TPA (10⁻⁷) induced down-regulation of p27^{KIP1} and up-regulation of cyclin D1 as early as within 5 h.

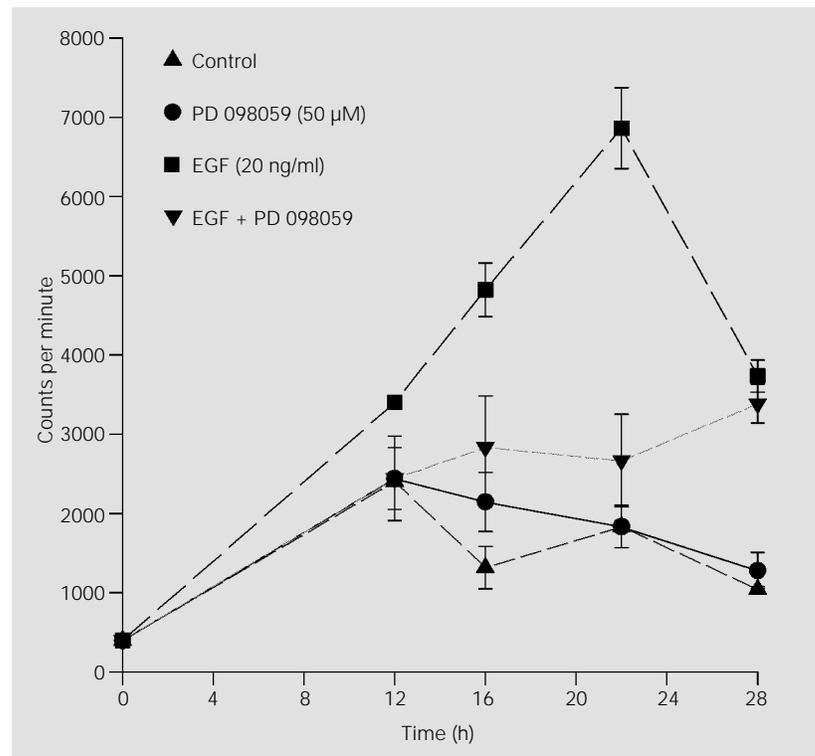


Figure 1 - Effect of PD 098059 on epidermal growth factor (EGF)-induced DNA synthesis. Postconfluent, quiescent C3H 10T1/2 cells were switched to Dulbecco's modified Eagle's medium (DMEM) containing 0.1% fetal bovine serum (FBS) 24 h prior to T = 0 h when 50 μ M PD 098059 or 20 ng/ml EGF was added. DNA synthesis was measured by 2-h pulse [³H]-thymidine incorporation at the indicated times.

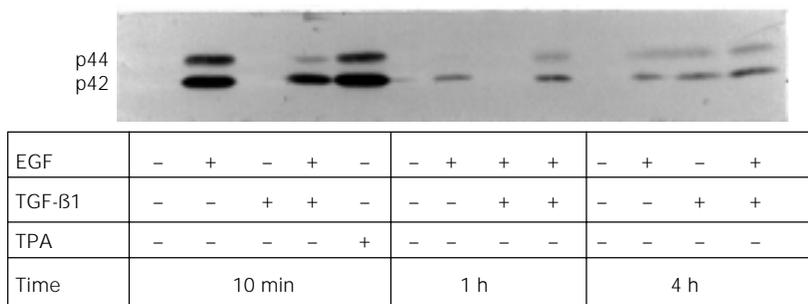


Figure 2 - Effect of TGF-β1 on epidermal growth factor (EGF)-induced p42/p44^{MAPK} activation. Postconfluent, quiescent C3H 10T1/2 cells were switched to DMEM containing 0.1% FBS 24 h prior to T = 0 h when 20 ng/ml EGF or 5 ng/ml TGF-β1 or 1 μM tetradecanoylphorbol acetate (TPA) was added. At the indicated times, whole cell extracts were prepared, subjected to SDS-PAGE on 12% gels, and transferred to nitrocellulose (14). Western blotting analysis was performed by probing the blots with a polyclonal antibody to the active, dually phosphorylated p42/p44^{MAPK} obtained from Promega Corp.

The up-regulation of cyclin D1 but not the down-regulation of p27^{KIP1} was sensitive to PD 098059 (10 μM). This was also correlated with the inhibition of PD 098059 on TPA-induced p42/p44^{MAPK} as well as DNA synthesis as observed by [³H]-thymidine incorporation. These studies agree with the findings of Weber et al. (17) who reported that overexpression of a dominant negative extracellular regulated kinase (ERK) resulted in the inhibition of PDGF-induced cyclin D1 expression but had no effect on PDGF-induced p27^{KIP1} degradation. In summary, these findings suggest that activation of the cell cycle by TPA as well as by EGF and PDGF involves the induction of p42/p44^{MAPK} activity followed by activation of the cyclin D1

promoter. Further, the data suggest that there is an independent regulation of cyclin D1 and p27^{KIP1} and the convergence of the p42/p44^{MAPK} pathway leading to cyclin D1 activation with another pathway responsible for p27^{KIP1} down-regulation establishes the activation state of complexes of cyclin-dependent kinases following mitogen treatment.

Since a dynamic balance exists between phosphorylation and dephosphorylation in the MAPK pathway resulting from an interplay between protein kinases and phosphatases, modification of either component is likely to have an important impact on signal transduction. Therefore, it is considered that since activation of p42/p44^{MAPK} requires dual phosphorylation on Thr and Tyr residues by MEK1/2, the protein phosphatases that inactivate MAPKs and MEKs must play critical roles in the level of activation of these enzymes *in vivo* but their identities are not clear. The catalytic subunit of protein phosphatase-1 (PP1) and protein phosphatase-2A (PP2A) inactivates MEK *in vitro*, with PP2A being much more effective (18). Also, a dual specificity MAP kinase phosphatase was found by the Tonks group to be highly specific for MAP kinase *in vitro* (19). Further studies are needed to learn whether TGF-β1 influences phosphatase activity which blocks EGF-induced p42/p44^{MAPK} activity.

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