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Close Correlation between the Dephosphorylation of p53 and Growth Suppression by Transforming Growth Factor-β1 in Nasopharyngeal Carcinoma Cells Transduced with Adenovirus Early Region Genes

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The mechanism of growth inhibition by transforming growth factor (TGF)-β1 was investigated. We examined the growth inhibitory effects of TGF-β1 on human nasopharyngeal carcinoma (KB) cells which constitutively expressed p53. TGF-β1 suppressed the DNA synthesis of KB cells in a dose-dependent manner. It had minimal effect on adenovirus-2-transduced KB cells expressing either adenovirus early region 1B (E1B) or 1A (E1A) product, which respectively binds to p53 or Rb product and inhibits its function, and no growth inhibition at all was observed with KB cells expressing both E1B and E1A products. Dephosphorylation of the p53 was promoted by TGF-β1 stimulation in KB cells, but not in E1B-producing KB cells, which sequestrate the function of p53. The growth inhibition of KB cells by TGF-β1 was significantly reduced by treatment with okadaic acid. These results suggest that p53 transduces the antiproliferative signal of TGF-β1 possibly through its dephosphorylation.

Key words: TGF-β1 — p53 — Adenovirus early region genes — Dephosphorylation

Transforming growth factor (TGF)-β1 is a growth inhibitory factor which exists in tumor cells as well as in certain normal cells,[1] and inhibits the passage to S phase in the cell cycle by acting on the late G1 phase.[2–4] This factor is known to inhibit cell division by suppressing the phosphorylation of retinoblastoma gene product (pRb)[3] or the expression of c-*myc*.[5–8] Another putative factor or factors in the signaling pathway of TGF-β1 has been suggested to exist on the basis of the fact that TGF-β1 suppresses the growth of cells without Rb expression[9] or down-regulation of c-*myc* expression.[10, 11] p53, like pRb, inhibits the expression of c-*myc*,[5, 7] and arrests the cell's passage from the late G1 phase to S phase.[12–15] However, transfection of a mutated p53 gene in some epithelial cell lines has been proved to nullify their responsiveness to TGF-β1.[16]

Based on these facts, we hypothesized that cell growth regulation by TGF-β1 and p53 may proceed by a common pathway and that p53 functions, as has been suggested by some other researchers, as a signal acceptor for TGF-β1. To test this hypothesis, we used KB-8, KB-18 and KB-16 derived from human nasopharyngeal carcinoma KB cells: these had been transfected with adenovirus-2 cDNA to express its early region 1A (E1A) gene product, early region 1B (E1B) gene product, and the products of both, respectively.[17] Since the E1A and E1B products bind to and thereby deactivate pRb[18] and p53,[19] respectively, it is plausible to expect that these transfectants would escape growth regulation by TGF-β1.

KB (a human nasopharyngeal carcinoma), KB-8 (KB transduced with E1A), KB-18 (KB transduced with E1B), and KB-16 (KB transduced with both E1A and E1B) were obtained from Yukijirushi Laboratories Inc., Tokyo. The cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, Gibco BRL, Gaithersburg, MD). Cells were incubated in the presence of 1–10 ng/ml human recombinant TGF-β1 (Nakarai, Kyoto) for 24 h in RPMI 1640 containing 2% FCS.

For Western blot analysis, cells were seeded at a density of 5×10^5 cells per 35 mm dish. After incubation for 24 h, cells were washed once with ice-cold 25 m*M* Tris-HCl, 150 m*M* NaCl, pH 8.0 and lysed with 50 m*M* Tris-HCl, 150 m*M* NaCl, 1% Triton X-100, 1 m*M* phenylmethylsulfonyl fluoride (PMSF), and 0.2 U/ml aprotinin, pH 8.0 for 30 min at 4°C. The lysates were cleared by microcentrifugation for 15 min at 4°C, the protein concentration of the supernatant was determined, and 100 μg aliquots of each lysate were analyzed by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Proteins in the gel were electrophoretically transferred onto Immobilon-P (Millipore) membranes. The membrane was probed with monoclonal antibody against Rb

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(Ab-2, Oncogene Science, Uniondale, NY) or p53 (Ab-2, Oncogene Science). The antigen-antibody complex was detected by using an ABC-kit (Vector Laboratories, Inc., Burlingame, CA).

A million cells were incubated with 10 ng/ml of TGF-β1 for 24 h and labeled for the last 3 h with 1 mCi/ml ^{32}P-orthophosphate (specific activity 9,000 Ci/mmol; ICN Biomedicals, Irvine, CA) in phosphate-free Dulbecco's modified essential medium (P^--DMEM) containing 0.5% dialyzed FCS. The labeling was terminated by three washes with P^--DMEM. Then cells were lysed with RIPA buffer (50 mM Tris-HCl, 120 mM NaCl, 0.5% NP-40, 100 mM NaF, 0.2 U/ml aprotinin, 1 mM PMSF, 10 μg/ml leupeptin, pH 8.0) for 30 min at 4°C. The lysate was collected and cleared by microcentrifugation for 20 min at 4°C, and the supernatant was recovered. Supernatant containing 500 μg of total cellular protein was incubated with 1 μg/ml of anti-p53 antibody, followed by 1 μg/ml rabbit anti-mouse IgG as described previously.[3] Immunoprecipitates were collected by absorption on protein A-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden). Protein A-Sepharose beads were washed five times with RIPA buffer, boiled in electrophoresis sample buffer in the presence of dithiothreitol, and subjected to 12.5% SDS-PAGE. The gel was fixed, dried and exposed to Kodak AR film at −70°C for 1 day.

Cells were seeded at a density of 1×10^4 cells per well into 24-well plates in RPMI 1640 medium supplemented with 10% FCS and incubated at 37°C for 24 h. They were then washed three times with RPMI 1640 medium and replaced with the same medium containing 2% FCS and TGF-β1 at the concentrations indicated. Cultures were incubated at 37°C for 20 h and pulsed for 4 h with 0.2 μCi/ml of ^{3}H-thymidine. Each well was then rinsed with phosphate-buffered saline, fixed with methanol for 10 min, dissolved in 0.1 N NaOH and heated to 60°C for 20 min. Radioactivity was determined by liquid scintillation counting. Assays were performed in triplicate, and results are represented as percentage (mean±SD) incorporation of radiolabel into DNA in comparison to untreated control cells.

Analysis of cell proliferation was performed by measuring mitochondrial function as an indicator of cell viability, e.g., by cleaving 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Promega, Madison, WI) to form a colored formazan product. The MTT test to assess cell viability was carried out by seeding 5,000 cells/well into 96-well plates for each condition, and cultures were incubated for 3 days. Following standard processing, the absorbance at 550 nm was measured with an enzyme-linked immunosorbent assay reader. The results were expressed as follows:

$$\frac{\text{corrected absorbance of test well}}{\text{untreated control well}} \times 100.$$

First, we examined p53 in these cell lines by Western blotting, and found more intense bands in KB-18 and KB-16 than in the parental KB cells (Fig. 1a). This was evidence that p53 had been stabilized by the formation of a complex with E1B protein (55 kd) in these cells: the presence of this complex was confirmed by immunoprecipitation followed by Western blotting with KB-18 and KB-16 cells (Fig. 1c). We also investigated the form of p53 (wild type or mutated) in these cells, because it has not previously been determined which types of p53 are capable of binding to the E1B protein. Single strand conformation polymorphism (SSCP) analysis revealed no abnormalities in highly conserved boxes II to V, which are known to contain hypermutation points in the

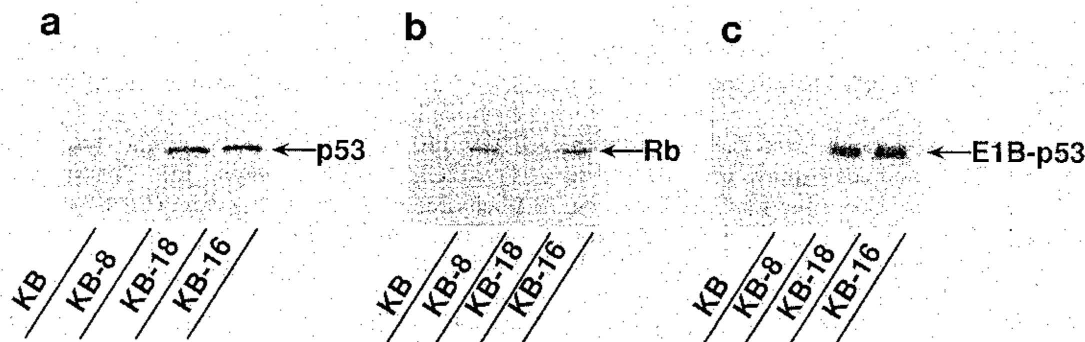

Fig. 1. Immunoblot analysis of p53, E1B bound p53, and pRb in KB, KB-8, KB-16, and KB-18 cells. Cell extract (100 μg) of each cell line was electrophoresed and transferred onto Immobilon-P membrane. To detect E1B-bound p53 in cells, cell extract was reacted with anti-E1B antibody immobilized Sepharose beads, and immunoprecipitated proteins were electrophoresed (c). The blots were probed with anti-p53 antibody (a, c), and with anti pRb antibody (b). The antigen-antibody complex was visualized by using ABC-kit as described in the text.

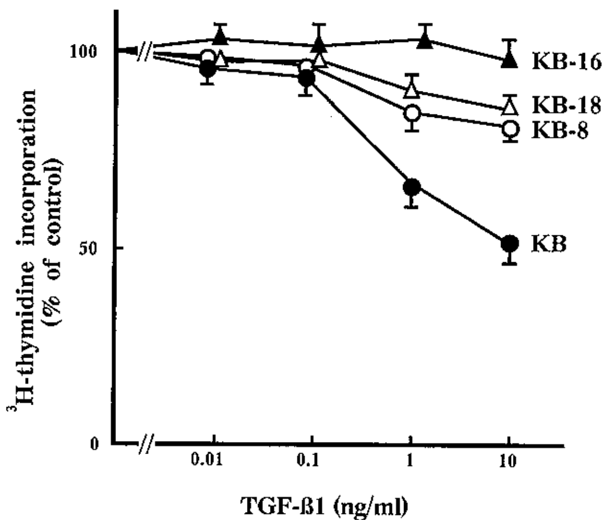

Fig. 2. Effect of TGF-β1 on DNA synthesis of KB, KB-8, KB-16, and KB-18 cells. Statistical analysis was performed by using Student's t test.

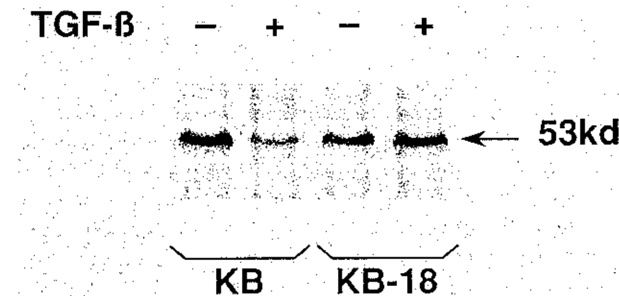

Fig. 3. Effect of TGF-β1 on p53 phosphorylation in parental KB cells and constitutively E1B gene-expressing KB cells. KB and KB-18 cells were incubated with or without 10 ng/ml of TGF-β1 for 24 h and labeled with ^{32}P-orthophosphate for the last 3 h.

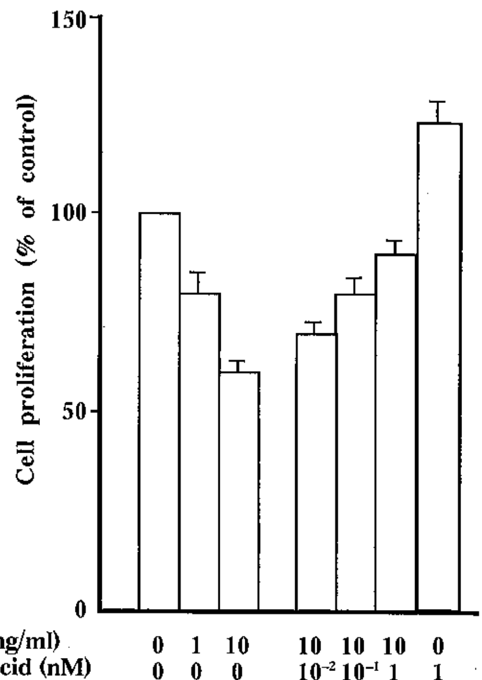

Fig. 4. Effect of a protein phosphatase inhibitor, okadaic acid, on the sensitivity of KB cells to TGF-β1. Cells were incubated with TGF-β1 in the presence or absence of okadaic acid for 72 h in 96-well microtiter plates. Cell viability was then determined by means of the MTT test.

p53 gene (data not shown). Expression of Rb in these cells was also examined: as expected, cells expressing E1A product (KB-8, KB-16) showed stronger expression of Rb on the Western blot (Fig. 1b), indicating E1A-Rb complex formation. The sensitivity of KB, KB-8, KB-18 and KB-16 cells to TGF-β1 was then determined. DNA synthesis in KB cells decreased to 50% of the initial level after incubation with 10 ng/ml of TGF-β1 for 8 h, and plateaued for at least 24 h (data not shown). Therefore, an incubation time of 24 h was chosen for the following experiments. Growth suppression at various concentrations of TGF-β1 was determined in these cells.

Dose-dependent suppression of DNA synthesis by TGF-β1 was clearly observed in KB cells, while sensitivities to TGF-β1 were significantly reduced in KB-8 and KB-18, and almost no sensitivity was observed in KB-16 (Fig. 2). These results are compatible with our hypothesis that KB cells become TGF-β1-insensitive upon transfection with the E1B gene, whose product sequesters p53 from the signaling pathway of TGF-β1. The above results are also consistent with an earlier report that cells became insensitive to TGF-β1 when they were transduced with the viral oncogenes SV40 large T antigen and E7 protein, which are known to bind to pRb.[4]

The nature of the growth suppression signals transduced by p53 is unknown. It is believed that, like pRb, p53 is involved at some point in the transcriptional suppression of the cell cycle-related genes c-*myc*[5, 7] and c-*fos*,[12] although the mechanism is unknown. Furthermore, linkage of the growth suppressive function with unphosphorylated p53 is currently receiving much attention; the more highly phosphorylated forms were seen during the S and G2/M phase of the cell cycle, whereas the underphosphorylated p53 forms were found in G1 phase and in the growth-arrested state.[20-22] Recently, it

was demonstrated that the inhibition of cell growth by p53 was mediated by cyclin-dependent kinase-interacting proteins 1 (cip1) or wild type p53-activated fragment 1 (WAF1).[23-25] The p53 protein was also found to be phosphorylated *in vitro* by cdc2 protein kinase in a cell cycle-dependent manner: this was taken to indicate that its antiproliferative activity is regulated by the phosphorylation state.[26] Recent studies have afforded contradictory results; two confirmed antiproliferative effects of phosphorylated p53,[27,28] while another found no such correlation.[29]

In this context, we further explored the effect of TGF-β1 on p53 phosphorylation in KB cells. p53 phosphorylation was inhibited by TGF-β1 stimulation in parental KB cells, but not in KB-18 cells, which constitutively express E1B (Fig. 3), whereas no remarkable change in the p53 protein level was observed by the metabolic labeling/immunoprecipitation method (data not shown). To prove the involvement of p53 dephosphorylation in the signaling pathway of TGF-β1, an experiment using a phosphatase inhibitor, okadaic acid, was carried out. As shown in Fig. 4, the TGF-β1 sensitivity of KB cells was reduced by the treatment with this inhibitor in a dose-dependent manner, indicating that growth inhibition by TGF-β1 is closely related to dephosphorylation of p53. We also found the phosphorylated p53 was increased by addition of a protein phosphatase inhibitor, okadaic acid, in a dose-dependent manner (data not shown). Accordingly, we conclude that TGF-β1 inhibits cell growth by suppression of p53 phosphorylation.

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REFERENCES

1) Derynck, R., Goeddel, D. V., Ullrich, A., Gutterman, J. U., Williams, R. D., Bringman, T. S. and Berger, W. H. Synthesis of messenger RNAs for transforming growth factors α and β and the epidermal growth factor receptor by human tumors. *Cancer Res.*, **47**, 707–712 (1987).
2) Howe, P. H., Draetta, G. and Leof, E. B. Transforming growth factor β1 inhibition of p34cdc2 phosphorylation and histone H1 kinase activity is associated with G1/S-phase growth arrest. *Mol. Cell. Biol.*, **11**, 1185–1194 (1991).
3) Laiho, M., Decaprio, J. A., Ludlow, J. W., Livingston, D. M. and Massague, J. Growth inhibition by TGF-β linked to suppression of retinoblastoma protein phosphorylation. *Cell*, **62**, 175–185 (1990).
4) Pietenpol, J. A., Stein, R. W., Moran, E., Yaciuk, P., Schlegel, R., Lyons, R. M., Pittelkow, R. M., Munger, K., Hoekey, P. M. and Moses, H. L. TGF-β1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell*, **61**, 777–785 (1990).
5) Coffey, R. J., Bascom, C. C., Sipes, N. J., Graves-Deal, R., Weissman, B. E. and Moses, H. L. Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor beta. *Mol. Cell. Biol.*, **8**, 3088–3093 (1988).
6) Fernandez-Pol, J. A., Talkad, V. D., Klos, D. J. and Hamilton, P. D. Suppression of the EGF-dependent induction of C-MYC proto-oncogene expression by transforming growth factor β in a human breast carcinoma cell line. *Biochem. Biophys. Res. Commun.*, **144**, 1197–1205 (1987).
7) Pietenpol, J. A., Holt, J. T., Stein, R. W. and Moses, H. L. Transforming growth factor β1 suppression of c-myc gene transcription: role in inhibition of keratinocyte proliferation. *Proc. Natl. Acad. Sci. USA*, **87**, 3758–3782 (1990).
8) Takehara, K., LeRoy, E. C. and Grotendorst, G. R. TGF-β inhibition of endothelial cell proliferation, alteration of EGF binding and EGF-induced growth-regulatory (competence) gene expression. *Cell*, **49**, 415–422 (1987).
9) Ong, G., Sikora, K. and Gullick, W. J. Inactivation of the retinoblastoma gene does not lead to loss of TGF-β receptors or response to TGF-β in breast cancer cell lines. *Oncogene*, **6**, 761–763 (1991).
10) Chambard, J. C. and Pouyssegur, J. TGF-beta inhibits growth factor-induced DNA synthesis in hamster fibroblasts without affecting the early mitogenic events. *J. Cell Physiol.*, **135**, 101–107 (1988).
11) Smeland, E. B., Blomhoff, H. K., Holte, H., Ruud, E., Beisks, K., Funderud, S., Godal, T. and Ohlsson, R. Transforming growth factor type β (TGFβ) inhibits G1 to S transition, but not activation of human B lymphocytes. *Exp. Cell Res.*, **171**, 213–222 (1987).
12) Ginsberg, D., Mechta, F., Yaniv, M. and Oren, M. Wild-type p53 can down-modulate the activity of various promoters. *Proc. Natl. Acad. Sci. USA*, **88**, 9979–9983 (1991).
13) Martinez, J., Georgoff, I., Martinez, J. and Levine, A. J. Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. *Genes Dev.*, **5**, 151–159 (1991).
14) Michalovitz, D., Halevy, O. and Oren, M. Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. *Cell*, **62**, 671–680 (1990).
15) Milner, J. and Medcalf, E. A. Temperature-dependent

switching between "wild-type" and "mutant" forms of p53-Val135. *J. Mol. Biol.,* **216,** 481–484 (1990).
16) Gerwin, B. I., Spillare, E., Forrester, K., Lehman, T. A., Kispert, J., Welsh, J. A., Pfeifer, A. M. A., Lechner, J. F., Baker, S. J., Vogelstein, B. and Harris, C. C. Mutant p53 can induce tumorigenic conversion of human bronchial epithelial cells and reduce their responsiveness to a negative growth factor, transforming growth factor β_1. *Proc. Natl. Acad. Sci. USA,* **89,** 2759–2763 (1992).
17) Babiss, L. E., Young, C. S. H., Fisher, P. B. and Ginsberg, H. S. J. Expression of adenovirus E1a and E1b gene products and the *Escherichia coli* XGPRT gene in KB cells. *J. Virol.,* **46,** 454–465 (1983).
18) Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A. and Harlow, E. Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature,* **334,** 124–129 (1988).
19) Sarnow, P., Ho, Y. S., Williams, J. and Levine, A. J. Adenovirus E1b-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54kd cellular protein in transformed cells. *Cell,* **28,** 387–394 (1982).
20) Marshall, C. J. Tumor suppressor genes. *Cell,* **64,** 313–326 (1991).
21) Nurse, P. and Bissett, Y. Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. *Nature,* **292,** 558–560 (1981).
22) Suzuki, K., Ono, T. and Takahashi, K. Inhibition of DNA synthesis by TGF-β1 coincides with inhibition of phosphorylation and cytoplasmic translocation of p53 protein. *Biochem. Biophys. Res. Commun.,* **183,** 1175–1183 (1992).
23) Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. and Elledge, J. The p21 cdk-interacting protein cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell,* **75,** 805–816 (1993).
24) El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. and Vogelstein, B. WAF1, a potential mediator of p53 tumor suppression. *Cell,* **75,** 817–825 (1993).
25) Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. p21 is a universal inhibitor of cyclin kinases. *Nature,* **366,** 701–704 (1993).
26) Bischoff, J. R., Friedman, P. N., Marshak, D. R., Prives, C. and Beach, D. Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. *Proc. Natl. Acad. Sci. USA,* **87,** 4766–4770 (1990).
27) Milne, D. M., Palmer, R. H., Campbell, D. G. and Meek, D. W. Phosphorylation of the p53 tumour-suppressor protein at three N-terminal sites by a novel casein kinase I-like enzyme. *Oncogene,* **7,** 1361–1369 (1992).
28) Ullrich, S. J., Mercer, W. E. and Appella, E. Human wild-type p53 adopts a unique conformational and phosphorylation state *in vivo* during growth arrest of glioblastoma cells. *Oncogene,* **7,** 1635–1643 (1992).
29) Nigro, J. M., Sikorski, R., Reed, S. I. and Vogelstein, B. Human p53 and CDC2Hs genes combine to inhibit the proliferation of *Saccharomyces cerevisiae. Mol. Cell. Biol.,* **122,** 1357–1365 (1992).