Inhibition of Cell Growth by Transforming Growth Factor β1 Is Associated with p53-Independent Induction of p21 in Gastric Carcinoma Cells

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Cell cycle regulators such as cyclins, cyclin-dependent kinases (cdks) and their inhibitors control the growth of cells. SDI1/CIP1/WAF1/p21 is a potent inhibitor of G1 cdks, whose expression is induced by wild-type p53. To elucidate the mechanism of growth inhibition by transforming growth factor β1 (TGFβ1), we examined the effect of TGFβ1 on the expression of p21, G1 cyclins and cdks by human gastric cancer cell lines. TGFβ1 induced p21 expression and subsequently suppressed cdk2 kinase activity, followed by a reduction in phosphorylation of the product of the retinoblastoma tumor suppressor gene in TMK-1 cells, which are responsive to TGFβ1. Coimmunoprecipitation analysis demonstrated that TGFβ1 increased the level of p21 protein present in complexes with cdk2. In contrast, TGFβ1 did not induce p21 in TGFβ1-resistant MKN-28 cells. TGFβ1 did not affect the levels of p53 mRNA and protein in TMK-1 and MKN-28 cells, which contain mutated p53 genes. These mutated p53 complementary DNAs, when overexpressed, failed to activate transcription from the p21 promoter. Furthermore, TGFβ1 caused a reduction in the steady-state level of cyclin A protein concomitantly with inhibition of cdk2 kinase activity in TMK-1 cells. These results suggest that the growth inhibition of tumor cells by TGFβ1 is associated with p53-independent induction of p21, subsequent suppression of cdk activity and a decrease in cyclin A protein in TMK-1 cells.

Key words: Gastric carcinoma cells — Cell growth inhibition — TGFβ — p21 — p53

Transforming growth factor β1 (TGFβ1) is a multi-functional polypeptide which is involved in cellular growth, cell adhesion, and differentiation.[1] TGFβ1 inhibits proliferation of a variety of normal epithelial cells, as well as carcinoma cells, by delaying or arresting progression through the late portion of G1.[2] However, the mechanism of the growth inhibition by TGFβ1 remains unclear.

Since cyclin-dependent kinases (cdks) phosphorylate retinoblastoma tumor suppressor gene (Rb) during the G1 phase and then release cells from growth inhibition,[3,4] the negative growth effects of TGFβ1 should be linked to its ability to convert Rb to the underphosphorylated form.[2] In MvlLu mink lung epithelial cells, TGFβ1 not only blocks cdk2 activity by reducing the stability of the cyclin E-cdk2 complex, but also inhibits cdk4 synthesis, leading to inhibition of Rb phosphorylation and arrest of G1 progression.[5,6] Inhibition of cyclin A expression by TGFβ1 has also been reported in this cell line.[7]

Recently, four different protein inhibitors for cdks have been identified: SDI1/CIP1/WAF1/p21,[8–10] p27[Kip1],[11–13] p16[INK4A],[14] and p15[INK4B].[15] The p21 protein binds to a variety of cdk2-cyclin complexes and inhibits their activity.[9] The expression of p21 is known to be increased in senescent cells.[8] The transcription of the p21 gene is directly promoted by wild-type p53 in response to DNA-damaging agents that trigger G1 arrest or apoptosis.[10, 16] However, since p21 was also inducible in p53-negative cells, p21 expression must be regulated by other factors.[17, 18] p27 was originally identified and purified as a heat-stable protein that inhibits cyclin E-cdk2 function in MvlLu cells arrested by TGFβ1 or contact inhibition.[11] p27 with significant sequence homology to p21 binds to and inactivates G1 cyclin-cdk complexes in a manner similar to p21.[12, 13] Overexpression of p21 or p27 in mammalian cells inhibits cell growth.[9, 12]

As to stomach carcinogenesis, abnormalities in the TGFβ1/receptor sysytem and cell cycle regulators are clearly involved. It was reported that 60% of gastric carcinomas contain p53 gene alterations.[19] The gene amplification of cyclin E and increased cdc2 kinase activity are frequently associated with gastric carcinomas.[20, 21] On the other hand, most gastric carcinoma cells express less type I receptor for TGFβ, resulting in escape from the growth inhibition by TGFβ1, although they commonly express TGFβ1.[22, 23] We have previously found that TGFβ1 can inhibit the growth of only one out of the seven gastric carcinoma cell lines. In the present study, we investigated the effect of TGFβ1 on the expression of two cdk inhibitors, p21 and p27, in TGFβ1-responsive

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and unresponsive human gastric carcinoma cells. The effect of TGFβ1 on the protein expression of various G1 cyclins and cdks was also examined.

MATERIALS AND METHODS

Cell culture Two cell lines derived from human gastric carcinomas were used. MKN-28 cell line, well differentiated adenocarcinoma, was kindly provided by Dr. T. Suzuki. TMK-1 cell line was established from poorly differentiated adenocarcinoma in our laboratory.[24] The mean population doubling times of MKN-28 and TMK-1 cells were estimated to be approximately 38.0 and 34.9 h, respectively.[24, 25] The growth of TMK-1 cells is inhibited by TGFβ1, whereas that of MKN-28 cells is not.[26] TMK-1 and MKN-28 cells do not contain wild-type *p53* gene, but have missense mutations (codon 173, valine to methionine and codon 251, isoleucine to leucine, respectively).[27] These cells were routinely cultured in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo) containing 10% fetal bovine serum (FBS; Whittaker Bioproducts, Walkersville, MD) under humidified 5% CO_2 in air at 37°C. They were grown to subconfluence in the medium described above.

For transfection assay, SW480 cells, a human colon carcinoma cell line which has been used to assess the activity of p53,[10, 28] were obtained from American Type Culture Collection (Rockville, MD). This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui).

RNA preparation and Northern blot analysis TMK-1 and MKN-28 cells were cultured in serum-free medium for 24 h and incubated for 1 or 3 h in the presence of 100 p*M* TGFβ1 (R&B Systems, Minneapolis, MN) without FBS. RNAs were extracted by the standard guanidinium isothiocyanate-cesium chloride method.[29] Ten μg of polyadenylated selected RNA was electrophoresed on 1.0% agarose gel containing 6% formaldehyde and blotted onto a nylon membrane filter (Bio-Rad Laboratories, Richmond, CA). The filter was baked for 2 h at 80°C under vacuum. Hybridization, using ^{32}P-labeled probe, washing, and autoradiography were performed as described previously.[30] A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (CLONTECH Laboratories, Inc., Palo Alto, CA) was used as an internal control.

DNA probes The 1.5 kilobase (kb)-human *p27* cDNA[13] insert from pBluescript II SK$^{+/-}$ was kindly provided by Dr. J. Massagué. The 2.1 kb-human *p21* cDNA[8] insert from pcDSRαΔ was kindly provided by Dr. A. Noda. The 1.8 kb-human *p53* cDNA[31] insert from pR4-2 was kindly provided by Dr. E. Harlow.

Western blot analysis Subconfluent cells were incubated for 15 or 24 h in the presence of 100 p*M* TGFβ1 with 10% FBS. Cells were extracted with lysis buffer containing 50 m*M* Tris-HCl (pH 7.4), 125 m*M* NaCl, 0.1% Nonidet P-40 (NP-40), 5 m*M* EDTA, 0.1 *M* NaF, 10 μg/ml leupeptin, 0.1 mg/ml trypsin inhibitor, 0.1 mg/ml aprotinin, and 50 μg/ml phenylmethylsulfonyl fluoride. The protein concentration was determined by using the Bradford protein assay (Bio-Rad Laboratories) with bovine serum albumin as a standard. Western blotting was carried out as described.[32] The lysates (100 μg) were solubilized in Laemmli's sample buffer by boiling and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electrotransfer onto a nitrocellulose filter (Schleicher & Schuell, Dasse, Germany). The filter was incubated first with an appropriate primary antibody and then with peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody (Medical and Biological Laboratories, Nagoya) in the secondary reaction. The immune complex was visualized using the ECL Western blot detection system (Amersham, Aylesbury, UK).

Antibodies Anti-cdk2, anti-cyclin A, anti-cyclin D1, and anti-cyclin E polyclonal antibodies were purchased from UBI (Lake Placid, NY). Anti-cdk4, anti-Rb (G3-245) polyclonal and anti-p21 monoclonal antibodies were purchased from Pharmingen (San Diego, CA). Anti-p53 polyclonal antibody was purchased from Novocastra Lab. Ltd. (Newcastle, UK). Anti-p27 polyclonal antibody[12] was kindly provided by Dr. T. Hunter.

Immunoprecipitation Immunoprecipitation and kinase assay were carried out as described[21] with slight modifications. The lysates were pre-cleared in lysis buffer with 25 μl of a 1:1 slurry of protein G Sepharose (Pharmacia Biotech, Uppsala, Sweden) for 1 h at 4°C and subsequently centrifuged. The supernatants were incubated with 5 μg of anti-cdk2 antibody or anti-cyclin E antibody (UBI) and 40 μl of the slurry of protein G Sepharose for 3 h at 4°C. Precipitates were collected by centrifugation and washed 5 times with lysis buffer for SDS-PAGE or kinase assay.

Histone H1 kinase assay Precipitates were washed 3 times more in kinase assay buffer (50 m*M* Tris-HCl, pH 7.4, 10 m*M* $MgCl_2$ and 1 m*M* dithiothreitol). The precipitates were then suspended in 35 μl of kinase buffer containing 6 μg of histone H1 (Sigma, St. Louis, MO, type III-S) followed by a 5 min preincubation at 30°C. Subsequently, 5 μl of 60 μ*M* [γ-^{32}P]adenosine 5′-triphosphate (ATP) solution (3 μCi) was added, and the kinase reaction mixture was incubated at 30°C for 20 min. The reaction was stopped by adding 20 μl of Laemmli's sample buffer and boiling. The samples were subjected to 12% SDS-PAGE, followed by autoradiography.

Quantitative analysis of autoradiograms Autoradiograms were quantitated by densitometric scanning with an Epson GT-8000 (Seiko Epson Co., Nagano).

Plasmid constructs The mutant *p53* cDNAs containing

the entire open reading frame were obtained from TMK-1 and MKN-28 cells, respectively, by reverse transcription-polymerase chain reaction as described.[27] These p53 cDNAs were inserted into the EcoR I sites of a mammalian expression vector "pCR"3 vector (Invitrogen, San Diego, CA). The resultant DNA molecules were introduced into the DH5α strain of Escherichia coli (Life Technologies Inc., Gaithersburg, MD) by transformation. The clones which had the p53 cDNA insert in the sense direction were selected. MSVC1 and MSVKH215 encode wild-type and mutant p53, respectively, as described.[33] The 2.4 kb Xba I fragment of WWP-luc,[10] the p21 promoter construct, was inserted into a chloramphenicol acetyltransferase (CAT) reporter construct, pTKCAT,[34] to create WWP-CAT2. MSVC1, MSVKH215 and WWP-CAT2 were kindly provided by Dr. K. Nose.

Transfection and CAT assay Transient transfections were carried out by the Lipofectin method (Life Technologies) according to the instructions of the manufacturer. SW480 cells were plated at a density of 2×10^5 cells/60-mm dish and grown overnight. Cells were cotransfected for 48 h with 0.5 μg of a p53 cDNA or control vector ("pCR"3) and 2.5 μg WWP-CAT2 using 30 μg of Lipofectin reagent. In addition, 0.5 μg of β-galactosidase (β-gal) reporter construct (pSVβ-gal; Promega, Madison, WI) was included to control for transfection efficiency. Total cell extracts were prepared and assayed for total CAT (CAT enzyme-linked immunosorbent assay kit, Boehringer Mannheim Biochemica, Mannheim, Germany), β-gal (Non-Isotopic Immunoassay for Beta-

Galactosidase Transfection Protein, Life Technologies) and total cellular protein (Bio-Rad Protein Assay). Fold induction was calculated by normalizing the amounts of CAT protein to β-gal and total protein levels and by comparing CAT protein levels in SW480 cells cotransfected with the wild-type or the mutant p53 constructs with those cotransfected with control vector.

RESULTS

TGFβ1 induces p21 mRNA and protein in TMK-1 cells

First we examined the effect of TGFβ1 on the mRNA expression of p21 and p27. As shown in Fig. 1, the expression of p21 mRNA by TMK-1 cells was evidently induced by incubation with TGFβ1 for 1 h. The level of p21 mRNA after a 1 h incubation was about 7 times the control. Although it gradually decreased, the level of p21 mRNA remained at about 3 times the control at 12 h (data not shown). MKN-28 cells had a higher steady-state level of p21 mRNA than TMK-1 cells, and showed no induction of p21 by TGFβ1. The expression of p27 mRNA was not altered by TGFβ1 in TMK-1 cells, while it was slightly decreased in MKN-28 cells.

Since p21 is a downstream target of p53, we next investigated whether TGFβ1 affects the levels of p53 mRNA or not, even though both cell lines contain mutated p53 genes. As expected, the expression of p53 mRNA by both cell lines was not altered by TGFβ1 as confirmed by densitometry (Fig. 1).

We further studied the effect of TGFβ1 on the level of p21 protein by Western blot analysis. p21 protein was not detected in the cell lines before TGFβ1 treatment (Fig.
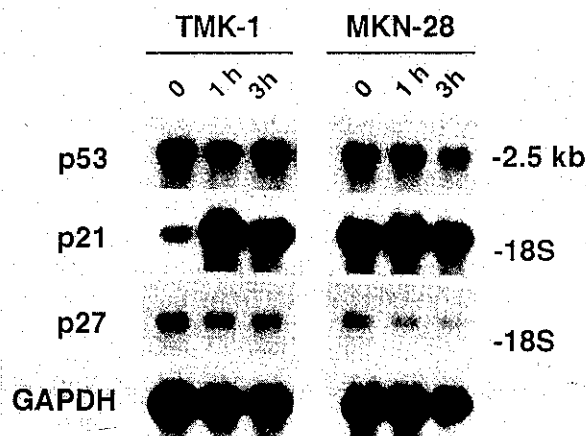

Fig. 1. Time course of changes of mRNA levels of p53, p21 and p27 in TMK-1 and MKN-28 cells after treatment with 100 pM TGFβ1 for the periods indicated. Polyadenylated selected RNA (10 μg) was subjected to Northern blot analysis as described in "Materials and Methods." A GAPDH probe was used as an internal control.
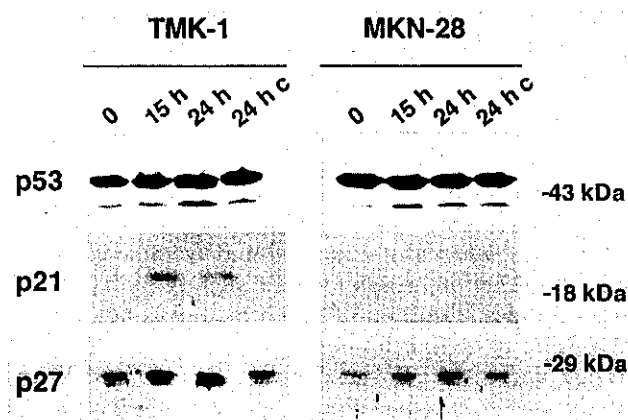
Fig. 2. Time course of changes of protein levels of p53, p21 and p27 in gastric carcinoma cells after treatment with 100 pM TGFβ1 for the periods indicated. Western blot analysis was performed on equal amounts of the cell extract as described in "Materials and Methods." 24 h c represents the treatment (−) control at 24 h.
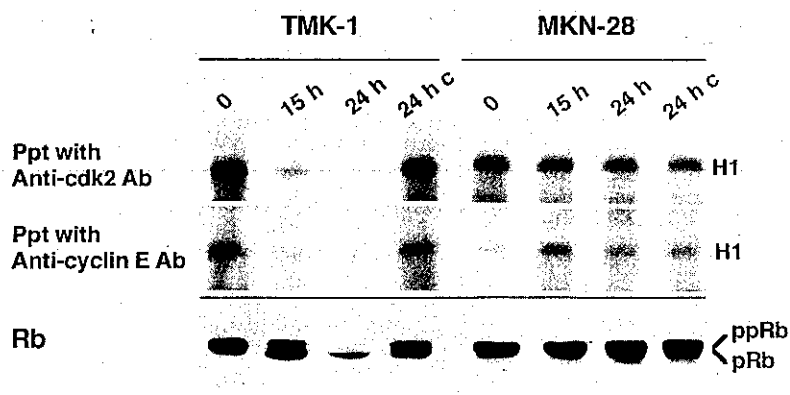
Fig. 3. Time course of changes of cdk2- and cyclin E-associated histone H1 kinase activity and Rb phosphorylation in gastric carcinoma cells after treatment with 100 pM TGFβ1 for the periods indicated. Either cdk2 or cyclin E immunoprecipitates from equal amounts of the lysates from subconfluent cells were assayed using histone H1 as a substrate. The reaction without the antibody or histone revealed no signal (data not shown). Western blot analysis was performed on the same amounts of the cell extract as in Fig. 2. ppRb and pRb indicate the hyperphosphorylated and underphosphorylated form, respectively. 24 h c represents the treatment (−) control at 24 h.
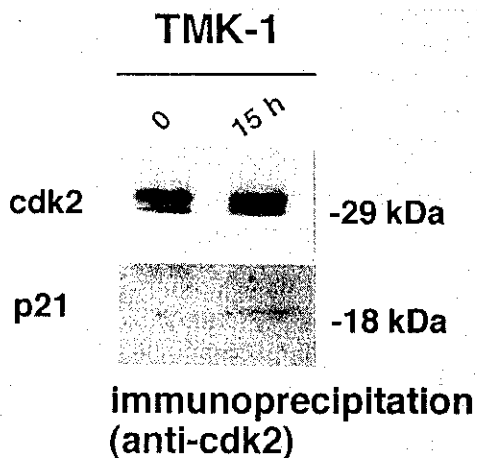

Fig. 4. Effect of TGFβ1 on the level of p21 present in complexes with cdk2 in TMK-1 cells. The cell extracts before TGFβ1 treatment and after treatment with 100 pM TGFβ1 for 15 h were first immunoprecipitated with antibody to cdk2 bound to protein G Sepharose prior to SDS-PAGE and detected by using anti-cdk2 and -p21 antibodies.

2). p21 protein was induced by TGFβ1 incubation for 15 h in TMK-1 cells, in accordance with the induction of *p21* mRNA, whereas it remained undetectable in MKN-28 cells. p27 protein was induced slightly by TGFβ1 in both cell lines. The level of p53 protein was not affected by TGFβ1 in these cell lines.

TGFβ1 decreases cdk2- and cyclin E-associated kinase activity and phosphorylation of Rb in TMK-1 cells We next examined the effect of TGFβ1 on cdk2- and cyclin E-associated kinase activity, because a major target of p21 is the cyclin E-cdk2 kinase complex.[9] Protein extracts from TGFβ1-treated and untreated cells were immunoprecipitated with anti-cdk2 or -cyclin E antibody and assayed for kinase activity using histone H1 as a

substrate. TGFβ1 reduced cdk2-associated kinase activity by 50% at 15 h and almost completely abolished it at 24 h in TMK-1 cells (Fig. 3). A similar inhibitory effect of TGFβ1 was detected on cyclin E-associated kinase activity. In MKN-28, no reduction of these kinase activities was observed.

The status of phosphorylation of Rb, a major target of cdk2,[4] was examined by Western blotting using the same protein extracts as used for the kinase assay. TGFβ1 reduced the level of phosphorylated Rb at 24 h in TMK-1 cells, whereas it did not affect the level in MKN-28 cells (Fig. 3).

TGFβ1 increases the level of p21 present in complexes with cdk2 in TMK-1 cells We examined whether the p21 protein induced by TGFβ1 is present in complexes with cdk2. Protein extracts were first immunoprecipitated with the cdk2 antibody bound to protein G Sepharose prior to SDS-PAGE. Probing with anti-p21 antibody showed a band migrating at Mr 21,000 (Fig. 4), indicating that TGFβ1 induces *p21* expression and increases the level of p21 present in complexes with cdk2.

TGFβ1 reduces the level of cyclin A in TMK-1 cells Recent studies have demonstrated that TGFβ1 suppresses expression of certain G1 cyclins or cdks in TGFβ1-responsive cell lines.[6, 7, 35, 36] In TMK-1 cells, the reduction of cdk activity could be implicated in inhibition of the synthesis of cyclins and cdks by TGFβ1. We then examined the effect of TGFβ1 on the protein expression of cdk2, cdk4, cyclin E, cyclin A and cyclin D1 by Western blotting. No obvious changes were detected in the expression of cyclins and cdks, except cyclin A in TMK-1 cells (Fig. 5). TGFβ1 caused an apparent decrease in cyclin A protein concomitantly with suppression of cdk2 kinase activity at 24 h in TMK-1 cells. We confirmed that cyclin A-associated kinase activity was very low at 24 h (data not shown).

Endogenous mutant p53 in TMK-1 cells can not activate *p21* promoter DNA damage causes a transcriptional
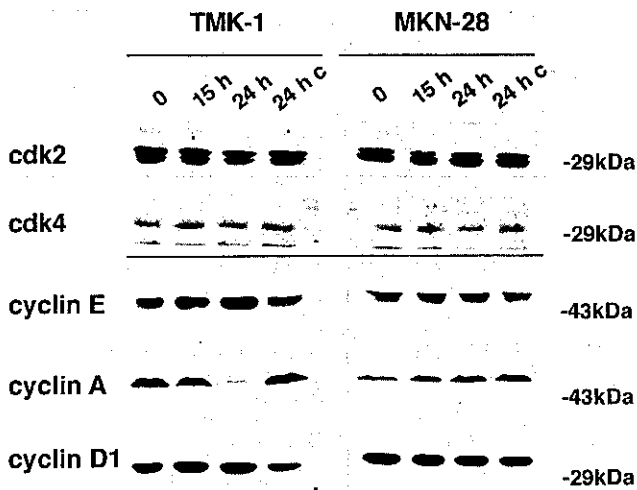
Fig. 5. Time course of changes of protein levels of cdks and G1 cyclins in gastric carcinoma cells after treatment with 100 pM TGFβ1 for the periods indicated. Western blot analysis was performed on the same amounts of the cell extract as in Fig. 2. 24 h c represents the treatment ($-$) control at 24 h.
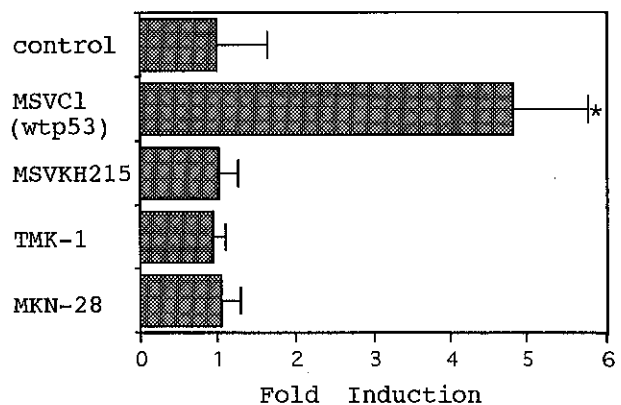

Fig. 6. Effect of endogenous p53 in two gastric carcinoma cell lines on *p21* promoter. Transcriptional activation was determined by cotransfecting 2.5 μg of the promoter constructs with 0.5 μg of the p53 expression constructs into SW480 and by measuring the amount of CAT. Fold induction was calculated by normalizing the amounts of CAT protein to β-gal and total protein levels and by comparing CAT protein levels in *p53* transfectants with those in cells cotransfected with control vector. Columns represent mean$\pm$SD (bar) of the results from 3 independent experiments. *, $P < 0.005$ vs. control (Student's t test).

activation of *p21* by functional p53.[10, 16] To investigate further the role of p53 in TGFβ mediated induction of p21, we examined the effect of TMK-1 endogenous p53 mutant on the *p21* gene promoter. The respective mutant *p53* cDNAs from TMK-1 and MKN-28 cells were ligated into the "pCR"3 vector and their transcriptional function was studied by cotransfection with the *p21* gene promoter-CAT reporter vector in SW480 cells and CAT assays. As shown in Fig. 6, wild-type *p53* transfectant (MSVC1) activated the *p21* promoter approximately 5-fold. In contrast, all mutant *p53* transfectants, including TMK-1 endogenous p53 mutant, exhibited almost the same level compared to the control (expression vector only). Western blot analysis showed that transfected mutant *p53* from TMK-1 had higher levels of protein expression than did the control (data not shown). Therefore, the mutant p53 from TMK-1 may fail to activate the *p21* promoter when overexpressed.

DISCUSSION

Our data demonstrated that TGFβ1 induced the expression of *p21* mRNA and increased p21 protein in complexes with cdk2, and subsequently inhibited cdk2 kinase activity and reduced the phosphorylation of Rb in TGFβ1-sensitive TMK-1 cells. Conversely, in TGFβ1-resistant MKN-28 cells, TGFβ1 did not induce *p21* expression. Therefore, the induction of p21 by TGFβ1 may be involved in the growth inhibition of TGFβ1 in TMK-1 cells.

The mechanism by which MKN-28 cells lose responsiveness to TGFβ1 remains obscure. Abnormalities at the receptor level might be involved. Recent reports have demonstrated that the TGFβ type II receptor gene is commonly mutated within a polyadenine tract in human colon and gastric cancer cell lines with microsatellite instability.[37, 38] However, our sequence analysis revealed that MKN-28 cells do not harbor the polyadenine mutation (Akagi and Yokozaki: unpublished observation). Further examination is needed to clarify the mechanism of escape from the growth inhibitory effect of TGFβ1 in this cell line.

p21 gene contains p53-binding sites with high homology to the p53 consensus motif.[10] p53 binds to different elements with different affinities, and missense mutations of *p53* have different effects on its ability to bind these elements and to activate transcription.[39-42] TMK-1 cells have a missense mutation of the *p53* gene at a highly conserved region which is presumed to contain amino acids essential for p53 function.[43] Therefore, we examined the transcriptional activity of TMK-1 endogenous p53 mutant. In transfection assay, this p53 mutant was unable to activate the *p21* promoter even if overexpressed. Furthermore, TGFβ1 did not affect the levels of p53 mRNA and protein in TMK-1 cells. Recently, Datto *et al.* reported that TGFβ1 interacts with *p21* promoter elements unrelated to p53.[44] These findings suggest that TGFβ1 induces p21 independently of the p53 pathway in this cell line.

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TGFβ1 signals

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┌─────────────────────────────────────────┐
│  p53 independent                         │
│                          ▼               │
│  ┌───┐ ↑            Cyclin A↓            │
│  │p21│                                   │
│  └───┘                                   │
│    │        ┌───┐                         │
│    │        │p21│                         │
│    ▼        └───┘          ▼             │
│  G1 cyclin/Cdk2     Cyclin A/Cdk2↓      │
│    │                        │             │
│    ▼                        │             │
│  Cdk2 kinase activity↓                   │
│       │                                   │
│       ┴                                   │
│  pRb ──┴──▶ ppRb                         │
│    │                                      │
└────│──────────────────────────────────────┘
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  Block cell cycle
  Growth inhibition
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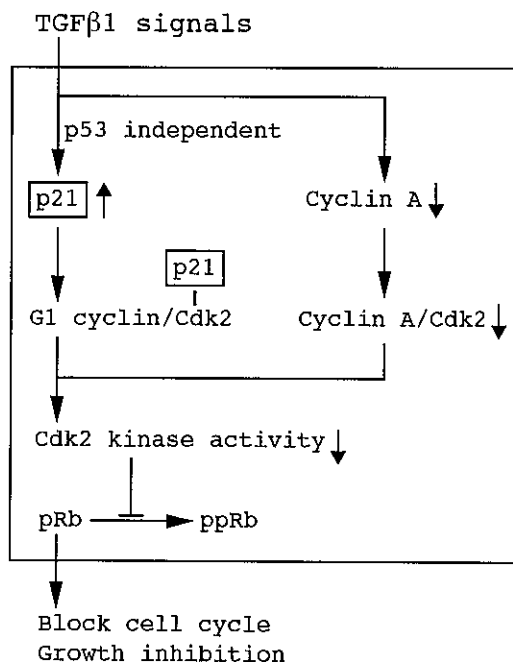
Fig. 7. Proposed mechanism of inhibition of cell growth by TGFβ1 in TMK-1 gastric cancer cells.

In TMK-1 cells, TGFβ1 evidently reduced the level of cyclin A protein. The reduction of cyclin A expression by TGFβ1 was also reported in other TGFβ1-sensitive cells.[7, 45, 46] In our study, however, it was not clear whether this reduction was the cause or the result of the growth inhibition by TGFβ1. In TMK-1 cells, the reduction of cyclin A protein was concomitant with the inhibition of cdk2 kinase activity at 24 h after the treatment. Cyclin A-associated kinase activity was also decreased at this point. Therefore, not only p21 induction, but also cyclin A reduction might decrease cdk2 kinase activity, resulting in the inhibition of Rb phosphorylation and growth suppression. Recently, Barlat et al. reported that cyclin A expression is down-regulated by TGFβ1 in Chinese hamster lung fibroblasts and this effect is mostly mediated at the transcriptional level through a cAMP-responsive element (CRE).[47] In TMK-1 cells, TGFβ1

reduced the expression of cyclin A at the mRNA level (data not shown). Therefore, the cyclin A cascade might be independent of the p21 cascade.

p27 was originally identified as a protein that inhibits cyclin E-cdk2 function in Mv1Lu cells arrested by TGFβ1, suggesting that p27 may be involved in TGFβ1-mediated inhibition.[11] However, in this study, TGFβ1 induced p27 expression only slightly in both TGFβ1-responsive and -unresponsive cell lines. In our gastric carcinoma system, p27 might not be involved in growth inhibition of TGFβ1.

Recently, it has been reported that in several cancer cells responsive to growth inhibition of TGFβ1, TGFβ1 causes an induction of p21 concomitantly with a reduction in cdk2 or cyclin E kinase activity.[48, 49] In OVCA420 ovarian cancer cell line, the reduction of cdk2 kinase activity was accompanied by a reduction of cdk2 protein.[48] However, in TMK-1 cells, TGFβ1 induced p21 protein and inhibited cdk2 kinase activity without a reduction of cdk2 protein. In addition, cdk4 protein levels remained constant after TGFβ1 treatment. On the other hand, in Mv1Lu cells, TGFβ1 significantly inhibits cdk4 synthesis and the inhibition of cdk4 activity leads to inhibition of cdk2 activation,[6] while cdk2 protein levels remain constant.[7] These findings suggest that TGFβ1 may affect cdk expression differently in different cell types.

In conclusion, a possible mechanism of growth inhibition by TGFβ1 in TMK-1 gastric cancer cells is shown in Fig. 7. p53-independent p21 induction and the decrease in cyclin A by TGFβ1 may play a pivotal role in growth inhibition of TMK-1. These events may result in inhibition of cdk2 kinase activity and Rb phosphorylation.

ACKNOWLEDGMENTS

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