Identification of Active Substances from *Streptomyces* Culture Fluids Using p53-Independent Expression of p21/WAF1/Cip1 Gene and Their Mode of Action

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An assay system was constructed to identify chemicals that have a potential to induce p21/WAF1 gene, a target of the tumor suppressor p53 critical for negative growth regulation. Screening of about 1300 culture fluids of *Streptomyces* resulted in identification of active substances which induced the p21 gene in a p53-independent manner; one was a mixture of four members of the actinomycin group, and the other was trichostatin A. Transcriptional regulatory regions of p21 gene for induction by actinomycin D and trichostatin A were determined by transient expression of luciferase constructs in cells which are p53-deficient (Saos-2) or express a mutated form of p53 (TMK-1). The essential transcriptional elements for the response to these drugs localize within 210 bp of the 5'-upstream region of human p21 gene, and Sp1 elements were determined to be critical for the induction. DNA-binding activity of Sp1 was not increased in cells treated with these drugs, but kinase inhibitors such as staurosporin and wortmannin inhibited the induction.

Key words p21 gene; screening; actinomycin D; trichostatin; Sp1

Negative regulation of cell cycle occurs when cells are exposed to external stresses such as DNA damaging agents, and cell cycle arrest is induced in either the G₁ or G₂ stages to prevent the cells from prematurely entering the next stage before the DNA is repaired.¹⁾ Recent studies indicate that the tumor suppressor p53 plays an important role as a cell cycle check point determinant following irradiation, and most human cancer cells that contain a mutated form of p53 did not show cell cycle arrest in response to DNA damage.^{2,3)} Negative control of cell cycle progression is important for the maintenance of genomic integrity, and many tumor cells seem to have lost this control mechanism.

p21/WAF1/Cip1 gene was identified as a target of p53 and also to encode a protein that inhibits the activity of cyclin-Cdk complexes, thereby negatively controlling the cell cycle progression.^{4–6)} The expression of p21 gene is, however, controlled by diverse mechanisms in a p53-dependent and -independent manner. Induction of p21 by γ -irradiation is a result of direct transcriptional activation by p53,^{7,8)} whereas various signals have been reported to induce p21 independent of p53, including serum, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), okadaic acid, 2-O-tetradecanoylphorbol 13-acetate (TPA), granulocyte colony stimulating factor (G-CSF), interleukin (IL)-6, interferon (IFN)- γ , and tumor growth factor (TGF)- β .^{9–15)}

A number of chemotherapeutic and DNA damaging agents such as alkylating agents, metal complexes, intercalating agents, vinca alkaloids, nucleosides and folate analogs have been shown to enhance wild-type p53 accumulation in the cell nucleus.¹⁶⁾ Evidence suggests that enhanced p53 levels are required for growth arrest in response to DNA damage, since cells lacking wild-type p53 do not exhibit increased p53 levels in response to DNA damage and do not exhibit growth arrest.¹⁷⁾ Although whether or not the DNA damage-induced p53-dependent G₁ arrest is mediated by WAF/p21/Cip1 remains to be investigated, it is known that the p21/WAF1/Cip1 gene product inhibits cyclin-dependent kinase ac-

tivity and blocks the cellular transition from G₁ to S.^{4,18)}

Among cyclin-CDK inhibitors, p16^{INK2} associates with cyclin D-cdk4,^{19,20)} while p27^{Kip1} is induced by TGF- β and cell-cell contact, and associates with cyclin E-cdk2, cyclin A-cdk2, cyclin D2-cdk4 complexes to inhibit activated complexes.²¹⁾ p15^{INK4B} is an inhibitor of cdk4 and cdk6 that associates with cyclin D.²²⁾ p21^{Cip1}, p27^{Kip1} and p57^{Kip2} inhibit essentially all G1/S CDKs.^{18,23)} Among these inhibitors, p21^{Cip1} (also known as WAF1) is modulated extensively as to expression. From this background, it was expected that substances that cause induction of these cdk inhibitors are potential anti-cancer agents, and p21 gene seemed the most promising target for screening of active substances. We have constructed screening systems to isolate active substances that increase the expression of p21 gene, and identified several known antibiotics. In the systems, human osteosarcoma (Saos-2) or gastric carcinoma (TMK-1) cells were stably transformed with luciferase reporters under transcriptional control of human p21 enhancers, and culture fluids of *Streptomyces* were tested. In the present communication, we describe two known drugs and their mode of action.

MATERIALS AND METHODS.

Construction of Reporters A luciferase reporter (WWP/Luc) that contained a DNA fragment –2400 bp upstream of human WAF1/p21 gene was a kind gift of Dr. K. W. Kinzler, The Johns Hopkins University.⁸⁾ A 210 bp *Pst*I–*Hind*III fragment that included a TATA box and cap site of WAF1/p21 gene was cut out from WWP/Luc and ligated with pGL2 basic vector (Promega) to construct WWP/Pst/Luc. Mutations were introduced into the *Pst*I–*Hind*III DNA fragment by PCR with oligonucleotides that contained mutations at three Sp1 or Ap-2 sites using a kit (Mutan, Takara, Kyoto). As a reference, the pRL-SV40 vector that expresses luciferase from *Renilla veniformis* was used.

Transfection of Plasmids and Luciferase Assay Human

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osteoblastic cells (Saos-2) that are p53-defective²⁴⁾ or human gastric carcinoma cells (TMK-1) that express mutant form of p53²⁵⁾ were transfected with luciferase constructs by a conventional calcium phosphate coprecipitation technique. Cells were plated at 1×10^4 cells/35 mm dish. After 1 d, cells were treated with 5 μ g/dish of reporter plasmid DNA in calcium phosphate precipitates for 4 h. Trichostatin-A, or actinomycin-D was added 8 h after the transfection, and 24 h after the transfection, cell lysates were collected for a luciferase assay. Luciferase activities from pGL2 and pRL-SV40 vectors were differentially measured in a single tube using specific assay conditions described in the manufacturer's manual (Promega Corp., Madison, WI). Luciferase activities of pGL2 constructs were normalized for those of pRL-SV40 in cell lysates. All the luciferase assays were carried out at least in triplicate, and each experiment was repeated at least three times. For isolation of stable transformants, the reporters together with selection vector, pSV2_{neo}, were transfected, and G418-resistant clones were isolated after selection for 3 weeks in the presence of 400 μ g/ml G418.

RNA Analysis Total RNA was extracted by the guanidium-hot phenol method,²⁶⁾ and 20 μ g of RNA per lane was separated on agarose gel containing 2.2 M formaldehyde. The RNA was then transferred to a nylon membrane (Hybond N, Amersham) and hybridized with ³²P-labeled probes in 50% formamide at 42 °C for 24 h. The molecular probe used was human WAF1/p21 cDNA (a gift from Dr. K. Kikuchi, Kanagawa Cancer Center). A fragment was cut out by digestion with *Xho*I and *Hind*III from the vector, and was labeled with ³²P-dCTP. After hybridization, filters were washed as described,²⁷⁾ and autoradiographed. As a reference, rat glyceraldehyde dehydrogenase (GAPDH) cDNA (GD5) was used to monitor the amounts of RNA.

Gel Mobility Shift Assay Nuclear extracts were prepared following the procedure of Dignam *et al.*²⁸⁾ The cells were collected with a rubber policeman, suspended in hypotonic buffer A (10 mM HEPES (*N*-hydroxyethylpiperazine-*N*,2-ethanesulfonic acid), pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 1 mM phenylmethyl-sulfonyl fluoride; PMSF, and 1 mM dithiothreitol; DTT), and centrifuged with a Dounce homogenizer. The nuclear fraction obtained by centrifugation was extracted with buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 1 mM PMSF, and 1 mM DTT) at 4 °C for 30 min. The extract collected by centrifugation was dialyzed against 50 $\times$ vol. of buffer D (20 mM HEPES, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, 1 mM PMSF, and 1 mM DTT) at 4 °C for 5 h. The dialysate was cleared by centrifugation, and aliquots were kept frozen at -80 °C. Sodium molybdate (10 mM) was added to all buffers to prevent phosphatase activity. The probes used were synthetic oligonucleotides for Sp1 sequence (5'-TGCAGGCCCGGGGAGGCGGTCCCGGGCGGCGCGG-3' and GATCCGCGCCCGGGGACCGCCCTCCCGGGGCC-3') subcloned into *Sma*I site of pUC18. The inserts were cut out with the restriction endonucleases and terminally labeled with ³²P-dCTP and Klenow fragments.

For the binding assay, nuclear extract (10 μ g of protein) was incubated in 24 μ l of binding buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 4 mM MgCl₂, 4 mM spermidine, 0.2 mM EDTA, 0.5 mM DTT, 0.05% (v/v) Nonidet P-40 (NP-40),

20% (v/v) glycerol, and 1 mg/ml of bovine serum albumin) containing 1 μ g of poly (dI-dC): poly (dI-dC), and competitor DNA (100 ng) as indicated for 1 h at 4 °C. The ³²P-labeled probe (0.2 ng, 5000 cpm) was added last, and the mixture was incubated for 30 min at room temperature. Samples were analyzed on native 4% polyacrylamide gel containing 6.7 mM Tris-HCl, pH 7.5, 3.3 mM sodium acetate, and 1 mM EDTA for 2 h at 11 V/cm. The gels were dried and autoradiographed with an intensifying screen at -70 °C.

Identification of Active Substances from Culture Fluids of *Streptomyces* Species *Streptomyces* species designated as MK643-101F3 and MK695-94F6 were detected to produce active substances. The active substances were purified from culture filtrates of each strain by successive extraction with ethyl acetate, silica gel chromatography, and repeated cycles of HPLC. From MK643-101F3, four active substances were purified: their structures were not determined, but all were assigned to the actinomycin group on the basis of UV, NMR, and MASS spectra. The actinomycin D provided by Sigma Chemicals, Co. (St. Louis) showed similar activity, and was used in subsequent experiments. The active substance from MK695-94F6 was determined as trochostatin A on the basis of UV, NMR, and MASS spectra and direct comparison by TLC with the authentic sample obtained from Sigma Chemicals.

RESULTS AND DISCUSSION.

Identification of p21-Inducing Activity in Culture Broth of *Streptomyces* Stable transformants of Saos-2 transfected with WWP/Luc or with WWP/Pst/Luc in combination with Neo^r-plasmid were isolated after selection in G418, and inducibility of luciferase was examined using 2 mM butyrate, a potent inducer of p21 gene.²⁹⁾ The clones that responded efficiently were isolated, and the clones Saos/#2 (transformed with WWP/Luc) and Saos/Pst#2 (transformed with WWP/Pst/Luc) were used for subsequent studies. These transformants responded to butyrate at levels comparable to those obtained in the transient assay (Fig. 1). We similarly established transformants of TMK-1 (TMK/#59 and TMK/Pst#22) that express a mutant form of p53 using WWP/Luc and WWP/Pst/Luc, respectively.

Using the above stable transformants, screening was carried out to identify substances that induced p21 gene expression. Among approximately 1300 culture fluids examined, two (MK643-101F3 and MK695-94F6) showed strong activity to induce WWP/Luc (Fig. 2). The induction is caused by p53-independent pathways, since both agents induced WWP/Luc as well as WWP/Pst/Luc that contained no p53-response element, and Saos-2 cells are p53-deficient. Active principles were purified and their structures were estimated by NMR and mass spectrometry. From MK643-101F3, four active compounds were isolated; all of them showed the same UV absorption spectra ($\lambda_{\max}$: 244 and 442 nm) as an actinomycin group. In PMR spectra, there were peaks thought to have originated from the chromophore of the actinomycin group (two coupled ring-protons and two ring-CH₃). Molecular peaks shown by mass spectra were not identical with the already known member of the actinomycin group. Instead of detailed research on their structure, p21-inducing activity of actinomycin D was determined and it was used for further

experiments. From MK695-94F6 trichostatin A was determined as an active substance.

Figure 3 shows the dose response to trichostatin A and actinomycin D of the stable transformants. Trichostatin A above 5 μM was toxic to the cells, but below this level it efficiently induced both WWP/Luc and WWP/Pst/Luc. Actinomycin D, an inhibitor of RNA synthesis, induced the luciferase activity at around 12 to 50 ng/ml. Clones from Saos-2 and TMK-1 responded similarly. These results suggest that the present systems are useful for the screening of biologically active substances including anti-cancer drugs.

Trichostatin A, and Actinomycin D Increased WAF1/Cip1/p21 mRNA in TMK-1 Cells To investigate whether

endogenous cellular WAF1/Cip1/p21 gene was induced by trichostatin A and actinomycin D, mRNA levels of WAF1/Cip1/p21 were examined by Northern blot analysis in TMK-1 cells. TMK-1 cells were used in this experiment, since the basal p21 mRNA level was unstable in Saos-2 cells. In the untreated control cells, WAF1/Cip1/p21 expression was too weak to be detected, probably due to the lack of wild-type p53 gene in the cells (Fig 4). The time course study showed that WAF1/Cip1/p21 mRNA was induced 3 h after the treatment with 4 μM trichostatin A or 25 ng/ml actinomycin D, and peaked at around 12 h after the treatment, when mRNA levels were induced approximately 5-fold compared with the control. The induction remained for at least 24 h. Trichostatin A and actinomycin D, on the other hand, could not activate other major cyclin-dependent kinase inhibitors such as p27/kip1 and p16/INK4A at the mRNA level (data not shown).

Fig. 1. Inducibility of Luciferase in Transiently Transfected Cells and Stable Clones

a; WWP/Luc was introduced transiently into Saos-2 cells, and the cells were either untreated or treated with 2 mM Na-butyrate for 16 h (lanes 1, 2, respectively). Lanes 3 and 4 are the activity of stable transformant Saos/#22 either untreated (lane 3) or treated with 2 mM Na-butyrate for 16 h (lane 4). b; WWP/Pst/Luc was introduced transiently into Saos-2 cells, and cells were either untreated or treated with 2 mM Na-butyrate for 16 h (lanes 1, 2, respectively). Lanes 3 and 4 are the activity of stable transformant Saos/Pst#22 either untreated or treated with 2 mM Na-butyrate for 16 h, respectively. Luciferase activity was measured, and the activity was normalized on the basis of protein of the extracts.

Fig. 2. WAF1/p21 Enhancer-Inducing Activity of Culture Broths and Their Extracts

From the first screening, two samples (MK643-101F3 and MK695-94F6) were found to contain strong inducing principles. Saos-2 cells that harbored WWP/Pst/Luc (#2) (hatched column) or WWP/Luc (#22) (dotted column) were treated with 1:100 (v/v) of culture fluid (lanes 2, 5) or their butyrate-extracts (lanes 3, 6) for 24 h, and luciferase activity was measured. Lanes 1 and 4 were untreated control, and lane 7 was the activity in cells treated with 2 mM Na butyrate.

Fig. 3. Dose Response of the Induction

Stable transformants of Saos-2 or TMK-2 were treated with indicated concentrations of actinomycin D or trichostatin A for 16 h, and luciferase activity was measured.

Thus, we conclude that trichostatin A and actinomycin D specifically induced WAF1/Cip1/p21 at the mRNA level.

Transcriptional Elements That are Responsible for the Induction

Fig. 4. Northern Blot Hybridization

TMK-1 cells were treated with 2 μ M trichostatin A (lanes 2–4) or with 25 ng/ml of actinomycin D (lanes 5–8) for 3 (lanes 2, 6), 6 (lanes 3, 7), 12 (lanes 4, 8) or 24 h (lane 5). Total RNA was extracted, and mRNA for p21 or glyceraldehyde dehydrogenase (GD5) was detected by hybridization.

ous signaling pathways. Since the WWP/Pst fragment contains no p53-response element, the induction by these chemicals is thought to be independent of p53 activation. This region is composed of several transcriptional regulators such as Sp1 elements, AP-2 element and TATA. Induction of p21/WAF1 gene by TPA and okadaic acid was reported to require the AP-2 element that localizes –105 to –95 bp of human p21/WAF1 gene,³⁰⁾ and a recent report showed that trichostatin A-mediated induction of the p21 gene depended on Sp1 sites.³¹⁾ To determine the elements responsible for actinomycin D and trichostatin in TMK-1 cells, luciferase reporters were constructed that had mutations in these elements. These constructs that express luciferase from fireflies were introduced into cells together with pRL-SV40 vector as an internal standard. The pRL-SV-40 constitutively expresses luciferase from Renilla that can be measured in a single tube in the presence of firefly luciferase under different assay conditions. The inducibility of each construct was measured in the absence or presence of chemicals, and the luciferase activity was normalized with the pRL-SV40 luciferase activity. The results of Fig. 5 indicate that induction by actinomycin

Fig. 5. Inducibility of Mutated Forms of WWP/Pst/Luc

Mutations were introduced at the underlined sites of the 5'-upstream region of human p21 gene (mutated sequences are shown below the lines), and DNA fragments were ligated to the luciferase reporter. Plasmids were introduced into TMK-1 cells. Cells were treated with 2 μ M trichostatin A, 2 mM Na-butyrate, or 50 ng/ml actinomycin D for 16 h, and luciferase activity was measured. Lane 1, untreated control. Lanes 1–6, WWP/Luc, WWP/Pst/Luc, mSP1-1, mSP1-2, mSP1-3, and mAP2, respectively. These reporters were transfected together with pRL-SV40, and the transfection efficiency was normalized with the luciferase activity from Renilla.+

Fig. 6. Sp1-Binding Activity of Nuclear Extracts from Cells Untreated or Treated with Trichostatin A or Actinomycin D

Nuclear extracts from control cells (lanes 1, 5), or cells treated with trichostatin A (4 μ M) (lanes 2–4) or actinomycin D (50 ng/ml) (lane 6) were mixed with labeled Sp1 probe, and the mixtures subjected to non-denaturing gel electrophoresis. Unlabeled Sp1 oligonucleotide (lane 3) or anti-Sp1 serum (lane 4) was included in the mixture.

Fig. 7. Effect of Kinase Inhibitors on the Induction by Trichostatin A or Actinomycin D

TMK/Pst#22 cells were either untreated (lanes 1–4), or induced with trichostatin A (4 μ M) (lanes 5–8) or actinomycin D (50 ng/ml) (lanes 9–12) in the absence (lanes 1, 5, 9) or presence of 250 nM wortmannin (lanes 2, 6, 10), 1 μ M genistein (lanes 3, 7, 11) or 0.1 μ M staurosporin (lanes 4, 8, 12) for 16 h. Luciferase activity was measured as described above.

D was dependent on Sp1 elements, but not on AP2 sites. Induction by trichostatin A was also dependent on Sp1 elements as reported by others.³⁰ Among three possible Sp1 elements, mutation at any one site abolished the induction.

Sp1-Binding Activity in Cells Treated with the Inducers The above results indicated that Sp1 elements were required for induction of p21 gene enhancer by actinomycin D or trichostatin A. Nuclear extracts were prepared from TMK-1 cells treated either with butyrate, trichostatin A, or actinomycin D for 16 h, and Sp1-binding activity was measured with ³²P-labeled synthetic oligonucleotide as a probe. The results in Fig. 6 show that the shifted band was specific for Sp1-binding activity, since the intensity of the band was reduced by homologous unlabeled oligonucleotide and the band was super-shifted in the presence of anti-Sp1. The Sp1-binding activity was not changed in cells treated with the inducers. It is speculated that Sp1-activation is necessary but

not sufficient for the induction of p21 gene.

Effect of Kinase Inhibitors on the Induction of WWP/Luc As DNA binding activity of Sp1 factor did not seem to be increased in cells treated with butyrate, trichostatin A or actinomycin D, signal transduction pathways may participate in the p53-independent induction of p21/WAF1 gene. To test this possibility, effects of kinase inhibitors on the induction by trichostatin A and actinomycin D were examined. The stable clone Saos/Pst#2 was treated in the presence or absence of wortmannin (PI3-kinase inhibitor),³² genistein (Tyr-kinase inhibitor) or staurosporin (Ser/Thr kinase inhibitor) for 16 hr, and luciferase activities were measured. Figure 7 shows that staurosporin strongly inhibited the induction, wortmannin inhibited it partially, and genistein had essentially no inhibitory effect. Trichostatin A is known as an inhibitor of histone deacetylase³³ like butyrate, and actinomycin D is an inhibitor of transcription. These agents with completely different actions induced p21 gene expression through apparently common transcriptional elements. The induction may be caused by activation of some signaling cascades elicited by cellular stress.

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