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IL-1 Induces Expression of p21^{WAF1} Independently of p53 in High-Passage Human Embryonic Fibroblasts WI38[1]

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We tested the effect of IL-1 on the expression of p21^{WAF1} in human embryonic fibroblasts WI38. Exposure to IL-1 caused induction of p21^{WAF1} protein in high-passage WI38 cells but not in early-passage cells. However, IL-1 did not stimulate the transcription of a CAT-reporter gene having two copies of the p53-responsive element on its promoter or the p53-binding capacity of nuclear extracts, although it increased transcriptional rate of p21^{WAF1} in these high-passage cells. These results suggest that the induction of p21^{WAF1} by IL-1 occurs at the transcriptional level, but p53 function is not required in these cells. Further studies found that IL-1 did not cause cell-cycle arrest, and the overexpression of p21^{WAF1} resulted in only a slight delay of cell growth, while the level of p21^{WAF1} coprecipitated with cyclin-dependent kinase-2 (Cdk2) was increased by IL-1. Moreover, a kinase assay of Cdk2 immunoprecipitates showed that IL-1 did not reduce the kinase activity, and IL-1 did not affect the status of phosphorylation of the retinoblastoma gene product (Rb). These findings imply that despite the induction of p21^{WAF1}, this cannot fully account for the growth arrest in high-passage WI38 cells. Thus, IL-1 mediates p21^{WAF1} induction through a p53-independent pathway(s) in high-passage WI38 cells, but the cell cycle is regulated independently of p21^{WAF1}.

Key words: fibroblasts, high passage, IL-1, independent of p53, p21^{WAF1}.

The synthesis and destruction of the cyclin partner oscillates markedly during the cell-cycle, but it can be induced by exogenous growth factors (*1–4*). The activated cyclin-dependent kinase (Cdk) can be inactivated by reducing the transcription or degradation of the protein itself (*5*). Recent studies, on the other hand, have identified a family of proteins with the ability to bind diverse cyclin-Cdk complexes and to inhibit their activity (*6*, *7*). These low molecular weight proteins, named cyclin-dependent kinase inhibitors (Ckis), are classified into two groups: the CIP/KIP family, which includes p21^{WAF1}, p27^{Kip1}, and p57^{Kip2}; and the INK4 family, which includes p15^{INK4b}, p16^{INK4a}, p18^{INK4c}, and p19^{INK4d}. p21^{WAF1} and p27^{Kip1} inhibit Cdk2-6, whereas p15^{INK4b} and p16^{INK4a} inhibit Cdk4 and Cdk6 (*6*, *7*). p21^{WAF1} is a p53-inducible gene that can inhibit the growth of human tumor cell lines and normal cells in the G1 phase (*8*, *9*). Studies have suggested that p21^{WAF1}-induced growth arrest involves direct inhibition of Cdk activity, which results in hypophosphorylation of Rb protein and accumulation of cells in the G1 phase of the cell-cycle (*10–13*).

p21^{WAF1} was the first Cki identified as a mediator of p53-induced growth arrest and as a direct Cdk regulator (*12*, *14–17*). Recent studies have elucidated other roles for p21^{WAF1}: it acts as an inhibitor of apoptosis as well as an inducer (*18*, *19*). The expression of the p21^{WAF1} gene is regulated by functional p53, which acts as a transcription factor controlling the arrest of cell-cycle progression at the G1 checkpoint in response to DNA damage. Thus, p21^{WAF1} is thought to be the main regulator of the G1 checkpoint control. However, the induction of p21^{WAF1} following stimulation by growth factor, during cellular differentiation or development, appears not to require p53 (*20*, *21*). p21^{WAF1} can be also regulated independently of p53 in p53-null cells exposed to radiation or cytokine (*20–28*).

Interleukin-1 (IL-1) is a novel proinflammatory cytokine that is produced by a variety of cells including macrophages/monocytes and fibroblasts, it has pleiotropic biological activities and is an important mediator in inflammation and hematopoiesis (*29*). It plays diverse biological roles in the host system, and its growth regulatory effect is cell-type-dependent: it stimulates the proliferation of cells such as thymocytes (*29–31*), while it inhibits the proliferation of various other cells (*32–35*). Events induced by IL-1 are receptor-mediated and dictated by immediate early gene expression events (*36–39*). IL-1 induces a number of immediate early genes in human cells. These include IRG-9, c-jun, NAK-1, MAD3, and Egr-1 (*36–39*). Egr-1 protein blocks the growth arrest action of IL-1 (*36*, *40*). Tumor necrosis factor (TNF) is a cytokine primarily produced by mono-

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cytes/macrophages (*40*, *41*). Many of the actions of TNF and IL-1 are similar or identical (*41*). TNF has an antiproliferative effect on various cells and causes G1/G0 arrest in the cell-cycle (*42*). Recently, we found that TNF induced expression of $p21^{WAF1}$ independently of p53 in cells including leukemic cells (*22*, *23*, *43*). Other cytokines such as epidermal growth factor (EGF), transforming growth factor-β (TGF-β), and interferon-γ (IFN-γ) have also been shown to induce $p21^{WAF1}$ expression in cells without the normal function of p53 (*24*, *44*, *45*).

Human diploid fibroblasts can be grown *in vitro* for a limited number of population doublings. $p21^{WAF1}$ was originally identified as a protein that was expressed at higher levels in senescent fibroblasts than in young cells (*17*, *46*). Further studies found that genes encoding proteins important for regulating the passage of cells through checkpoints within the cell-cycle had altered levels of expression in cells approaching senescence (*47*, *48*). There is also accumulative evidence that the cell-cycle and the expression of its related genes in senescent or high-passage fibroblasts are regulated through mechanisms different from those in early-passage cells (*49–51*). On the other hand, recent reports have shown that the contribution of $p21^{WAF1}$ to the cell-cycle arrest depends upon the cell type or external insults (*52*, *53*). Thus, it remains unclear whether the cell-cycle arrest is indeed mediated by $p21^{WAF1}$ in cells. Our present study showed that IL-1 induced $p21^{WAF1}$ expression in high-passage WI38 cells and investigated the mechanisms of the induction of $p21^{WAF1}$ in these cells Our data suggest that the regulation of the cell-cycle progression may be independent of $p21^{WAF1}$ and occur by a p53-independent mechanism in high-passage WI38 cells.

MATERIALS AND METHODS

Cells and Cell Culture—Normal human embryonic lung fibroblasts (WI38, American Type Tissue Culture Collection, Rockville, MD) were cultured in α-MEM (Cosmo Bio, Tokyo). The medium was supplemented with 7% fetal calf serum (FCS; Mitsubishi Kasei, Japan). The cells were incubated at 37°C in a 5% CO_2 atmosphere. In all experiments, early-passage (young) cells were between 25 and 30 mean population doublings (MPDs) and high-passage cells were used at over than 80 MPDs. For serum stimulation, cells were grown until they reached 80% confluence and then starved in α-MEM without FCS containing 30 nM Na_2SeO_4 and 5 μg/ml of human transferrin (WAKO, Osaka). After either 48 h, cells were stimulated by adding FCS.

Reagents and Antibodies—Recombinant human IL-1β (rhIL-1β, Genzyme, Cambridge, MA, Lot No: B 3632) had a specific activity of 5.0×10^8 U/mg protein. 12-*O*-Tetradecanoyl phorbol 13-acetate (TPA) was purchased from Sigma Chemicals (St. Louis, MO) and dissolved in acetone. To characterize proteins by Western blotting and immunoprecipitation of Cdk complexes, the following antibodies were used. p53 [PAb1801 (Ab-2)], $p21^{WAF1}$ [EA10 (Ab-1)], cyclin A [BF683 (Ab-2)], and cyclin D1 [HD11 (Ab-2)] monoclonal antibodies were purchased from Calbiochem (Cambridge, MA). Anti pRb (G3-245) and anti $p27^{Kip1}$ (G173-524) monoclonal antibodies were purchased from Pharmingen (San Diego, CA). Polyclonal antibodies specific for cyclin E, Cdk2, and Cdk4 were purchased from Upstate Biotechnology (UBI, Lake Placid, NY). Polyclonal antibody specific for Cdk6 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-$p16^{INK4a}$ (DCS-50.1/H4) or anti-SV40 large T antigen (PAb 416) monoclonal antibody was purchased from the Calbiochem. Alkaline phosphatase–conjugated goat anti–mouse IgG and anti rabbit IgG secondary antibodies were purchased from Gibco BRL (Gaithersburg, MD) and Zymed (San Francisco, CA), respectively.

DNA Probes—The pCEP4 plasmid containing human $p21^{WAF1}$ cDNA (2.1 kilobases: kb, *Not*I) was kindly provided by Dr. B. Vogelstein (The Johns Hopkins University, Baltimore, MA) (*14*) and β-actin DNA probe (0.7 kb, *Eco*RI–*Bam*HI) was from the plasmid of pHFβ A-3'ut (*54*). These probes were radiolabeled using a random primer DNA labeling kit (Megaprime® DNA labeling system, Amersham, Arlington Heights, IL) with ^{32}P-α-dCTP (3,000 Ci/mmol; Amersham).

Western Blot Analysis—Cells were lysed in buffer [50 mM Tris-HCl, pH 8.0, 150 mM sodium azide (NaN_3), 0.1% SDS, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1% Nonidet P-40 (NP-40), and 0.5% sodium deoxycholate]. After centrifugation, the protein concentrations of the lysates were measured by the method of Bradford (*55*). Equal amounts of cell lysates in SDS-PAGE sample-loading buffer [125 mM Tris-HCl (pH 6.8), 0.4% SDS, 20% glycerol, 2% β-mercaptoethanol, 1% bromophenol blue] were electrophoresed in polyacrylamide gels (6–15% acrylamide), then transferred to PVDF membranes (Immobilon®, Millipore, Bedford, MA). Membranes were incubated with each antibody. After washing, secondary antibodies conjugated with alkaline phosphatase were added to the blots. Immunoreactivity on blots was detected by NBT/BCIP (Gibco BRL) staining. Equal loading of the protein was verified by reprobing the blots with an anti-human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) antibody purchased from Trevigen (Gaithersburg, MD).

Isolation and Blotting of RNA—WI38 cells were suspended in hypotonic buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM NaCl, 3 mM $MgCl_2$, and 0.65% NP-40. Cytoplasmic RNA was extracted by the phenol/chloroform method as described (*56*). After denaturation at 65°C, RNA was electrophoresed in an agarose gel, containing formaldehyde (17%) and transferred to a nylon-membrane filter (Hybond N®, Amersham). Filters were hybridized with ^{32}P-labeled probe for 16 to 24 h at 42°C in hybridization buffer (50% formamide, 2× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate), 5× Denhardt's, 0.1% SDS, 10% dextran sulfate, 1 mM EDTA, and 100 μg/ml salmon sperm). Filters were washed to a stringency of 0.1× SSC at 65°C for 10 min and exposed to Fuji X-ray film (Fuji Photo Film, Tokyo).

Blots were usually sequentially hybridized with $p21^{WAF1}$ and β-actin radiolabeled probes. The actin band helped to confirm that similar amounts of RNA were added to each lane. Modulations in relative levels of $p21^{WAF1}$ RNA were quantified by standardization to the amount of β-actin specific transcripts. For a quantitative analysis, the radioactivities of bands of hybridization for β-actin mRNA and $p21^{WAF1}$ mRNA in the different lanes were quantified by BAS 2000® (Fuji). The ratio of $p21^{WAF1}$/β-actin in the control lane was assigned the baseline value. The fold-stimulation in the experimental lanes was calculated by multiplying the ratio of activity of $p21^{WAF1}$/β-actin transcripts by the reciprocal of the ratio of the baseline level.

Cell-Cycle Analysis—Cells were harvested by trypsinization, washed in ice-cold PBS, and fixed in 70% ethanol. After treatment with RNase A (0.1 μg/ml, 30 min at 37°C, Sigma), DNA was stained with propidium iodide (50 μg/ml, Sigma). Cell-cycle determination was performed using a fluorescence-activated cell analyzer, FACScan® (Becton-Dickinson, San Jose, CA). Percentages of cells in each cell-cycle phase were calculated using the software CellFIT® Cell-Cycle Analysis ver. 2.0.2 (Becton-Dickinson).

Detection of Cdks Associated p21^{WAF1} Protein by Immunoprecipitation—For examining Cdks associated with p21^{WAF1}, WI38 fibroblasts were washed with PBS twice and incubated in lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP-40, 50 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na_3VO_4), 1 mM PMSF, 25 μg/ml leupeptin, and 25 μg/ml aprotinin at 4°C for 30 min. Then cells were scraped free with a rubber policeman, collected, and sonicated on ice. Centrifuge-cleared cell extracts were subjected to immunoprecipitation with anti-Cdk2, -Cdk4, or -Cdk6 rabbit polyclonal antibody at 4°C for 2 h, then incubated for another 1 h with immobilized protein A–Sepharose beads (Pharmacia Biotech, Piscataway, NJ). The beads were washed with lysis buffer, centrifuged and resuspended in lysis buffer. The immunoprecipitated protein was separated by SDS-PAGE, and immunoblotting was performed using anti p21^{WAF1} antibody as described above.

Cyclin-Dependent Kinase Assay—For Cdks activity assays, Cdk2, Cdk4, and Cdk6 were immunoprecipitated as described above. Immunoprecipitates were washed four times with kinase assay buffer [50 mM HEPES-KOH (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT)]. The kinase reactions were initiated by resuspending the beads in 30 μl of kinase buffer containing 30 μM lithium ATP (Pharmacia), 10 μCi [γ-^{32}P]ATP (3,000 Ci/mmol; ICN), and 0.3 μg/μl histone H1 (Ambion, Austin, TX) as a substrate. After incubation at 30°C for 20 min, the reaction was terminated by the addition of SDS sample buffer, and the sample was boiled. The samples were separated in 10% SDS-PAGE. Then, the gel was dried and subjected to autoradiography. Relative intensities of bands were quantified by laser densitometry (Pharmacia).

Transcriptional Run-on Assay—The nuclear run-on assay was performed as described (*22*, *43*). WI38 fibroblasts were exposed to IL-1, and nuclei were isolated by suspension in a hypotonic buffer (10 mM Tris-HCl; pH 7.4, 10 mM KCl, 3 mM $MgCl_2$) and lysed by addition of 0.5% NP-40. Nuclei were harvested and washed in a hypotonic buffer containing 0.5% NP-40, then resuspended in nuclear storage buffer [40% glycerol, 50 mM Tris-HCl (pH 8.3), 5 mM $MgCl_2$, 0.1 mM EDTA]. Nuclei were incubated at 30°C for 30 min in a reaction buffer (150 mM KCl, 3 mM $MgCl_2$, 0.25 mM ATP, 0.25 mM GTP, 0.25 mM CTP) containing 200 μCi [α-^{32}P]UTP (3,000 Ci/mmol; ICN, Irvine, CA). The reaction was terminated by adding 300 units of DNase I (10 min, at 30°C). The reaction mixture was digested with 40 μg/ml of proteinase K in a solution containing 10 mM EDTA and 1% SDS, then phenol/chloroform extraction performed. The aqueous phase was precipitated at −70°C with an equal volume of isopropanol in the presence of 0.3 M sodium acetate (pH 6), and the precipitate was collected by centrifugation and dissolved in TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. After denaturation in 0.2 M NaOH (ice-cold) and neutralization in 0.3 mM HEPES, nuclear RNA was run through a Sephadex G50 spin column to remove unincorporated [^{32}P]UTP. Plasmid DNA containing the cDNA coding inserts was denatured by heat and alkalization (0.3 M NaOH). Denatured plasmids (5 μg for p21^{WAF1}, 2 μg for β-actin, and 2 μg for pUC19) were bound to nylon membranes (Hybond-N®) using BIO-DOT SF® (BioRad, Hercules, CA) and immobilized by UV cross-linker. The plasmid pUC19 was used to detect non-specific hybridization. Newly elongated nuclear RNA was hybridized to the filters containing plasmids. Hybridizations were performed with 10^7 c.p.m. of ^{32}P-labeled RNA/ml in 3× SSC, 5 mM EDTA, 0.1% SDS, 10× Denhardt's solution, 10% formamide, 10 mM NaH_2PO_4 (pH 7.0), 200 μg/ml of yeast tRNA, and 100 μg/ml of salmon sperm DNA for 3 days at 42°C. After hybridization, filters were rinsed in 2× SSC at room temperature, then in 2× SSC and 0.1× SSC at 42°C. The relative activity of bands of hybridization of p21^{WAF1} and β-actin in untreated and IL-1 treated lanes was quantified by BAS 2000®.

Plasmids—A reporter plasmid containing p53-binding sites was constructed in pBLCAT2 as described (*57*): two copies of the palindromic oligonucleotide 5′-TCGACGGA-CATGCCCGGGCATGTCCG-3′ were made and double-stranded by self-annealing, then cloned into the *Sal*I site of pBLCAT2 upstream of the enhancerless thymidine kinase (TK) promoter of Herpes Simplex Viruses. For the expression of p53, the cDNA of wild-type p53 was cloned into the pcDNA1 containing cytomegarovirus (CMV)-promoter as reported previously (*57*).

Overexpression of p21^{WAF1}—For the overexpression of p21^{WAF1}, the full length of human p21^{WAF1} cDNA was introduced into high-passage WI38 cells using an expression vector, pCEP4, driven by CMV-promoter (Invitrogen, Carlsbad, CA) (*14*). Forty-eight hours after transfection by electroporation, 0.2 mg/ml of hygromycin B (Sigma) was added to these cells, and cells were selected in the presence of hygromycin B for 2 to 3 weeks (*58*).

CAT Assay—Fifteen micrograms of reporter plasmid was transfected into WI38 cells by electroporation. Cells were cultured for 24 h after transfection, washed with culture medium, and then each reagent was added. After incubation for 24 h, cells were rinsed with cold PBS and scraped free with a rubber policeman. Cell lysate was made by sonicating cells in 100 μl of 0.25 M Tris-HCl (pH 7.8), and debris was removed by centrifugation. Ten microliters of a solution containing 4.3 mM acetyl CoA (Sigma) and 0.05 μCi [^{14}C]chloramphenicol (specific activity, 27 mCi/mmol, ICN) in 0.25 M Tris-HCl (pH 7.8) was added to 40 μl of the lysate, and the reaction mixture was incubated at 37°C for 2 h. The acetylated chloramphenicol products labeled with ^{14}C were assayed by TLC, and radioactivity was quantified by BAS 2000® (Fuji).

Gel Shift Assay—The preparation of nuclear extracts and the gel shift assays were performed as described (*59*). After treatment with IL-1, WI38 cells were scraped into ice-cold PBS, collected, and washed in ice-cold PBS. Cells were pelleted by centrifugation, then resuspended and incubated in hypotonic buffer (20 mM HEPES, 10 mM KCl, 1 mM EDTA) containing 0.2% NP-40, 10% glycerol, 1 mM DTT, 100 μM Na_3VO_4, and protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin) at 4°C for 15 min. Nuclei were collected by centrifugation (at 4°C, 8,000 ×*g*) and resuspended in ice-cold extraction

buffer (20 mM HEPES, 35 mM NaCl, 1 mM KCl, 1 mM EDTA) containing 20% glycerol, 1 mM DTT, 100 μM Na_3VO_4, and protease inhibitors, incubated at 4°C for 60 min with gentle shaking, then centrifuged at 4°C for 5 min (8,000 ×*g*). The supernatant was stored at −80°C until use. The protein concentration of the supernatant was measured by the method of Bradford. Binding reactions were carried out by incubating 5 μg of nuclear extract with a ^{32}P-end–labeled 20-mer double-stranded consensus p53-binding oligonucleotide from the human p21^{WAF1} promoter, 5′-GAACATGTCCCAACATGTTG-3′, in binding buffer containing 40 mM HEPES-KOH (pH 7.9), 50 mM KCl, 0.2 mM EDTA, 20% glycerol, 4 mM $MgCl_2$, 1 mM DTT, 0.05% NP-40, 4 mM spermidine, and 100 ng of poly (dI-dC) (Pharmacia) for 30 min at 4°C (*14*). Samples were separated by electrophoresis on a 5% polyacrylamide gel containing Tris glycine/EDTA (TGE) buffer. The specificity of binding was examined by competition with unlabeled oligonucleotide and standard protein. The dried gel was visualized by autoradiography.

RESULTS

IL-1 Induces p21^{WAF1} Expression in High-Passage Human Embryonic Lung Fibroblasts WI38 but Not in Early-Passage Cells—The expression of p21^{WAF1} and p53 by IL-1 in total cellular protein was examined in early- and high-passage WI38 fibroblasts by Western blot analysis. To determine whether IL-1 affects the expression of p21^{WAF1}, we cultured these WI38 cells with IL-1 at a concentration of 100 U/ml and examined the kinetics of p21^{WAF1} expression (Fig. 1, A and B). Cells were harvested sequentially at different time points following the addition of IL-1. As reported previously, p21^{WAF1} protein levels were relatively constant during the cell-cycle in both early- and high-passage cells, while slightly decreased levels of p21^{WAF1} protein was observed at 18 and 24 h of culture in untreated early-passage cells (Fig. 1A) (*60*). No changes in p53 levels were observed in these cells. Treatment of the cells with IL-1 did not affect levels of p21^{WAF1} or p53 protein. In high-passage cells, on the other hand, the levels of p21^{WAF1} protein markedly increased after 3 h of exposure to IL-1; the maximum level of p21^{WAF1} was observed after 6 h of exposure (Fig. 1B). These levels gradually decreased by 8 h, but the level of p21^{WAF1} after 24 h of IL-1 stimulation was still higher than at 0 h. Treatment of high-passage cells with IL-1 did not affect the levels of p53 protein.

IL-1 Increases Levels of p21^{WAF1} mRNA—To study the

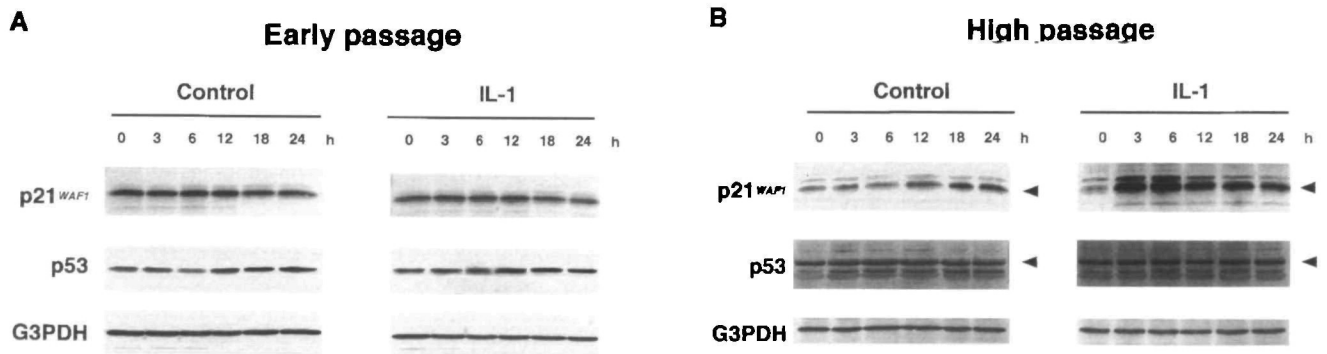

Fig 1. **IL-1 stimulates the accumulation of p21^{WAF1} in high-passage human embryonic fibroblasts WI38 but not in early-passage cells.** For induction of p21^{WAF1} protein by IL-1, fibroblasts were exposed to 100 U/ml of IL-1 for various periods. Cells were harvested, and whole cellular protein was electrophoresed in 12% polyacrylamide–SDS gel, then transferred to PVDF membrane. Western blot analysis was performed using either anti-p21^{WAF1}, anti-p53, or anti-G3PDH antibody as described in "MATERIALS AND METHODS."

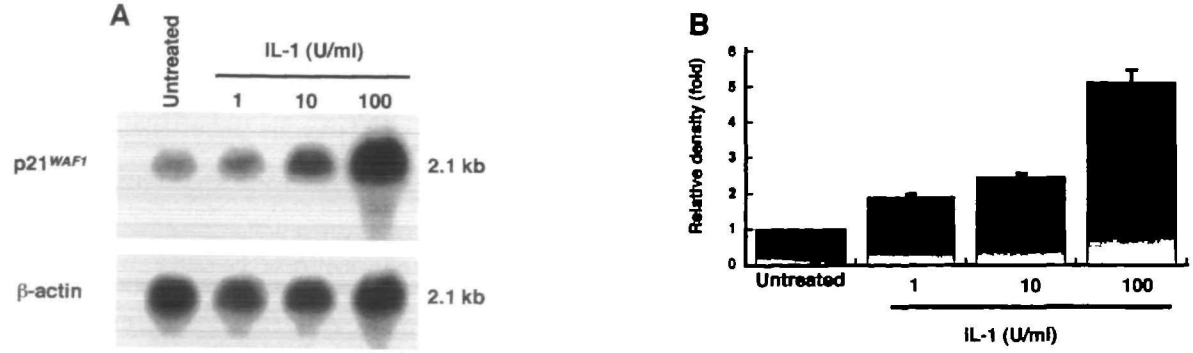

Fig 2. **IL-1 induces expression of p21^{WAF1} mRNA in high-passage fibroblasts.** Cells were cultured with various concentrations of IL-1 for 8 h, then cytoplasmic RNA was extracted. The level of p21^{WAF1} mRNA was determined by Northern blotting (A). The density of bands of hybridizaton of p21^{WAF1} and β-actin mRNAs was scanned by BAS 2000® (B). The modulation of relative levels of p21^{WAF1} mRNA was quantified with normalization to the amount of β-actin–specific transcripts. Results are presented as means of triplicate determinations (mean ± SE)

induction of p21^{WAF1} transcripts by IL-1 in high-passage cells, cells were cultured for 8 h with various concentrations of IL-1 and subjected to Northern blotting (Fig. 2A). Figure 2B shows a quantitative presentation of p21^{WAF1} mRNA levels in high-passage WI38 cells. These fibroblasts constitutively contained low concentrations of mRNA coding for p21^{WAF1}. IL-1 stimulated the accumulation of p21^{WAF1} mRNA in a dose-dependent manner; an increase in the level of p21^{WAF1} mRNA was induced by 1 U/ml of IL-1 (about 2-fold). Maximum levels of p21^{WAF1} mRNA occurred at 100 U/ml of IL-1, and the relative density was approximately five times greater than that of control. These fibroblasts exhibited constitutive expression of p53 mRNA. However, treatment with IL-1 did not increase the levels of the expression of p53 (data not shown).

Effects of IL-1 on the Cell-Cycle Progression in High-Passage WI38 Cells—To determine the effect of IL-1 on the cell-cycle progression in these fibroblasts, flow cytometric analysis of cells stained with propidium iodide was performed (Table I). High-passage WI38 cells were synchronized by serum deprivation for 48 h, then placed in fresh medium containing 7% serum. Deprivation of serum induced the G1 arrest in these cells; more than 80% of cells entered the G1 phase of the cell-cycle, while 50% of cells were in the G1 phase before serum-starvation. When cells were released from the block by serum deprivation, cells started to enter the cell-cycle and almost 75% of the cells had progressed to S phase by 18 h. The cells had started to divide by 24 h, and reentered G1 phase gradually. When synchronized cells were stimulated with IL-1 (100 U/ml) in the presence of 7% serum, no effects of IL-1 were observed on the cell-cycle as compared to the flow cytometric profiles of control and IL-1–treated cells, which were able to progress into S phase while p21^{WAF1} protein levels were significantly increased, as shown in Fig. 1B. In parallel, starved cells were cultured in medium with serum for 12 h, and then IL-1 was added to these cells at G1/S transition. However, no effects of IL-1 on the cell-cycle were observed in these cells.

Effects of IL-1 on the Kinase Activities of Cdks—We showed the induction of p21^{WAF1} by IL-1 in high-passage human embryonic fibroblasts. However, the increased levels of p21^{WAF1} protein did not affect the cell-cycle progression, as presented in Table I. To gain insight into the function of p21^{WAF1} induced by IL-1 during the cell-cycle progression, cell lysates from cells treated with or without IL-1 (100 U/ml) were immunoprecipitated with anti-Cdk2, -Cdk4, or -Cdk6 antibody. These immunoprecipitates were electrophoresed and immunoblotted with anti p21^{WAF1} antibody (Fig. 3A). Upon treatment with IL-1, the level of p21^{WAF1} protein coprecipitated with anti-Cdk2, -Cdk4, or -Cdk6 antibody was increased as compared to that of untreated cells. Furthermore, the kinase activity associated with the Cdk2 immunoprecipitates from the same cell lysates was determined by using histone H1 as a substrate (Fig. 3B). Treatment with IL-1 did not affect the kinase activity in these cells, while TPA inhibited the activity by 40% relative to the control (positive control for inhibition). IL-1 did not inhibit on the Cdk4 or Cdk6 kinase activity (Fig. 3C).

Phosphorylation of the Retinoblastoma Gene Product in IL-1–Treated Cells—The retinoblastoma gene product (Rb) is a major regulator of G1/S transition; the phosphorylation of endogenous Rb is important for the regulation of cell-cycle progression (*61*). Previous studies have shown that

TABLE I **Cell-cycle progression of high-passage WI38 fibroblasts treated with IL-1.**

Incubation duration	Cell cycle phase	Control	Treatment	
			IL-1	IL-1(+12 h)
		(% of cells)		
Untreated	G0/G1	52 7		
	S	31.7		
	G2/M	15 6		
Serum-starved	G0/G1	80 6		
	S	4.2		
	G2/M	15.2		
12 h	G0/G1	66.9	72 2	64.8
	S	14.5	10	16.9
	G2/M	18 5	17.8	18.2
18 h	G0/G1	22.6	19.2	27 5
	S	74 4	78 6	71 6
	G2/M	3	2.2	0 9
24 h	G0/G1	29 7	35 9	34.8
	S	33 3	28 9	30 4
	G2/M	37	35 2	34 8
36 h	G0/G1	49 6	36 1	42 4
	S	33 5	42 4	34 8
	G2/M	16.9	21.5	22 8
48 h	G0/G1	57.1	54	50.4
	S	21.4	25.6	32 3
	G2/M	21 5	20 4	17 3

High-passage WI38 cells were cultured in α-MEM without FCS for 48 h The serum-starved cells were stimulated with 7% FCS in the absence or presence of IL-1 at 100 U/ml In parallel, serum-starved cells were cultured with α-MEM with 7% FCS for 12 h, and then 100 U/ml of IL-1 was added. Cells were harvested at the indicated time and the nuclear DNA was stained with propidium iodide (PI). Cell-cycle progression was analyzed by flow cytometry The results were analyzed with the software CellFIT® Cell-Cycle Analysis Ver 2.0 2 (BECTON DICKINSON)

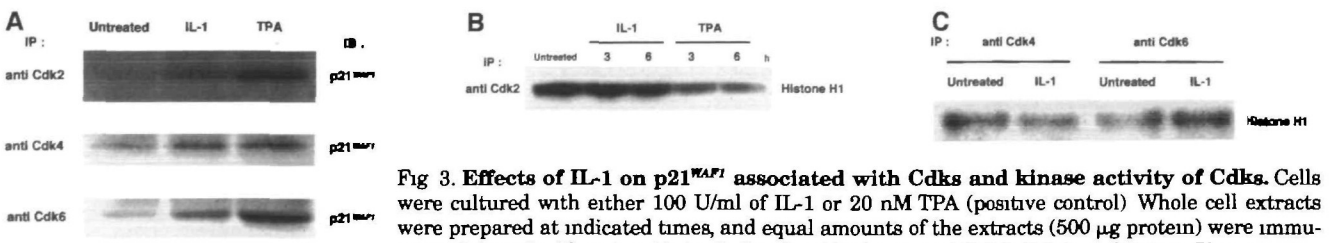

Fig 3. **Effects of IL-1 on p21^{WAF1} associated with Cdks and kinase activity of Cdks.** Cells were cultured with either 100 U/ml of IL-1 or 20 nM TPA (positive control) Whole cell extracts were prepared at indicated times, and equal amounts of the extracts (500 μg protein) were immunoprecipitated with anti-rabbit polyclonal antibody against Cdk2, Cdk4, or Cdk6 A. The immunoprecipitates (6 h) were separated by 12% SDS–polyacrylamide gel electrophoresis and transferred to PVDF membrane Immunoblot analysis with anti p21^{WAF1} antibody was performed B and C: Immunoprecipitates were also subjected to a kinase assay using histone H1 as a substrate and analyzed by 10% SDS-PAGE.

the IL-1–induced cell-cycle arrest is mediated by hypophosphorylation of Rb protein, which leads to growth arrest in the G1 phase of the cell-cycle (*36*, *38*, *39*). Hypophosphorylated forms of Rb ($p110^{Rb}$) migrate faster than hyperphosphorylated forms ($pp110^{Rb}$) in Western blot analysis (*62*). We examined the phosphorylation status of Rb in IL-1–treated fibroblasts. High-passage fibroblasts were serum-starved for 48 h as described above, then cultured with or without IL-1 for various periods in the presence of FCS and analyzed by Western blotting (Fig. 4). In serum-starved cells, the hypophosphorylated form of Rb ($p110^{Rb}$) predominated. In parallel with the entry of a fraction of these cells into S phase on stimulation by FCS, increased levels of the hyperphosphorylated form of Rb ($pp110^{Rb}$) were observed with concomitantly reduced levels of the hypophosphorylated form. At 36 h after stimulation with FCS, the hypophosphorylated Rb appeared; Rb hypophosphorylation was evident 48 h after the addition of FCS. These results are consistent with the fact that Rb protein is hypophosphorylated in non-proliferating cells. On the other hand, treatment with IL-1 did not affect the phosphorylation status of Rb in these cells.

Expression of Other Cell-Cycle–Associated Proteins in Cells Treated with IL-1—To study the mechanisms behind the lack of inhibition by IL-1–induced $p21^{WAF1}$, we examined the effects of IL-1 on the expression of the cell-cycle–associated proteins Cdk 2, Cdk 4, cyclin E, and cyclin D1, and other Cdk inhibitors, $p27^{Kip1}$ and $p16^{INK4a}$. Figure 5 presents the levels of $p27^{Kip1}$ at different stages of the cell-cycle either in the presence or absence of IL-1 determined by Western blotting. As the cell-cycle progressed, a transient decrease in the $p27^{Kip1}$ level was observed in the control, then the levels returned to baseline. On the other hand, treatment of WI38 cells with IL-1 decreased the accumulation of $p27^{Kip1}$ protein in an almost time-dependent manner. There were no effects of IL-1 on levels of Cdk2, Cdk4, cyclin D1, cyclin E, $p16^{INK4a}$, and cyclin A or E immunoprecipitated with Cdk2 as compared to untreated cells (data not shown).

Effect of Overexpression of $p21^{WAF1}$ on the Growth of High-Passage WI38 Cells—To further study the role of $p21^{WAF1}$ in the cell growth of high-passage WI38 fibroblasts, we transfected a $p21^{WAF1}$-expression vector into high-passage WI38 cells, selected several hygromycine B–resistant clones, and characterized them for basal $p21^{WAF1}$ expression relative to a control vector stable transformant (pCEP4).

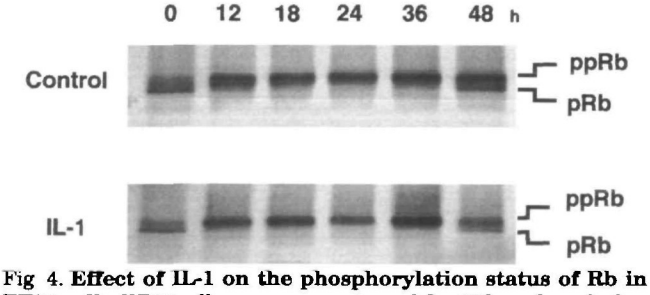

Fig 4. **Effect of IL-1 on the phosphorylation status of Rb in WI38 cells.** WI38 cells were serum-starved for 48 h as described in "MATERIALS AND METHODS," then cultured with or without 100 U/ml of IL-1 for the indicated time periods in the presence of FCS and analyzed by Western blotting Equal amounts (30 μg of protein) were loaded onto a 6% SDS–polyacrylamide gel, and immunoblotting was performed using anti-Rb protein antibody.

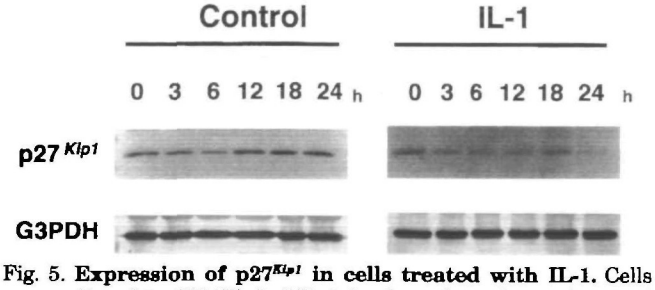

Fig. 5. **Expression of $p27^{Kip1}$ in cells treated with IL-1.** Cells were cultured in 100 U/ml of IL-1 for the indicated periods Equal amounts of protein (100 μg) were loaded onto a 12% SDS–polyacrylamide gel, separated, and analyzed by Western blotting. Equal loading of the cell extracts was confirmed by assaying the membrane with anti-G3PDH antibody (lower lane)

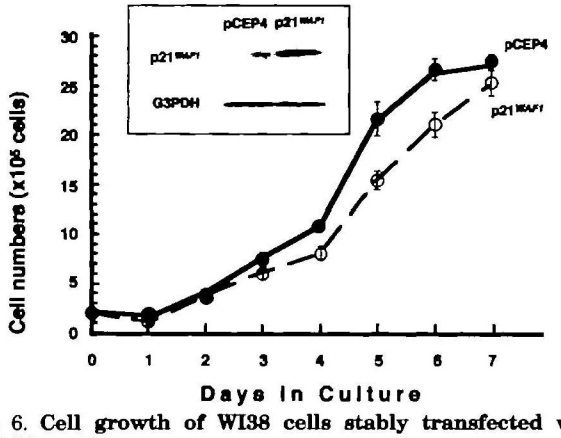

Fig. 6. **Cell growth of WI38 cells stably transfected with $p21^{WAF1}$.** High-passage WI38 cells were transfected with 15 μg of either a control vector (pCEP4) or an expression vector of $p21^{WAF1}$ Stable clones were obtained by hygromycin B selection (0.2 mg/ml) Total cellular proteins were harvested, and Western blot analysis was performed as described in "MATERIALS AND METHODS" (Inset) Cells transfected with pCEP4 (control) (●) or $p21^{WAF1}$ (○) were seeded at 2×10^5 cells and cultured Cells were collected on the indicated day by trypsinization and enumerated The graph shows the mean values for four separate experiments.

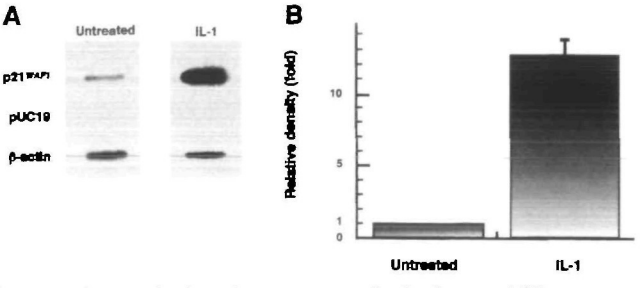

Fig 7. **Transcriptional run-on analysis for $p21^{WAF1}$ in cells treated with IL-1.** WI38 cells were either untreated or stimulated with IL-1 (100 U/ml) for 2 h. Nuclei were isolated as described in "MATERIALS AND METHODS." Newly elongated RNAs were hybridized to the plasmid containing either $p21^{WAF1}$ cDNA (5 μg) or β-actin DNA (2 μg) (A). The density of bands of $p21^{WAF1}$ or β-actin was quantified by BAS2000®, and the ratio of $p21^{WAF1}$/β-actin in untreated cells was compared to that in IL-1 treated cells (B). The results from three separate experiments are summarized

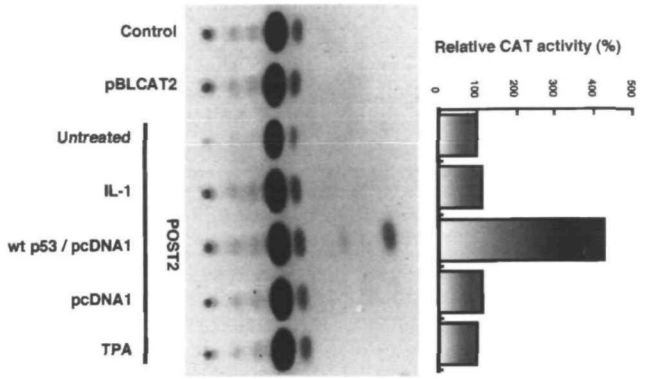

Fig. 8. **Effect of IL-1 on the CAT activity of the reporter gene containing the p53 responsive elements.** WI38 cells were transfected with 15 μg of CAT reporter plasmid containing the p53 response element (POST2) by electroporation As a positive control, the p53 expression vector (wt p53/pcDNA1) was co-transfected with POST2 To exclude non-specific activity in the assay system, cells were transfected with a control vector pBLCAT2 After overnight culture, IL-1 (100 U/ml) or TPA (20 nM, negative control) was added to the cells. Cells were incubated for 24 h, then CAT assay was performed Results are expressed as the percent CAT activity relative to untreated cells (transfected with POST2)

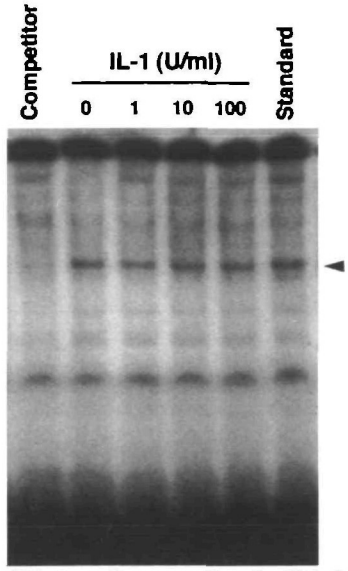

Fig 9 **Effects of IL-1 on the capacity of p53 to bind DNA.** Cells were incubated with different concentrations of IL-1 (1-100 U/ml) for 1 h, then nuclear extracts were prepared After incubation of the nuclear extracts with ^{32}P-end–labeled oligonucleotides of the p53-binding sequence from the p21^{WAF1} promoter at 4°C for 30 min, gel shift assay was performed. The arrow shows the band of complexes of probe and protein. To detect the specificity of the formation of oligonucleotide/protein complexes, a competition assay of the nuclear extract of untreated cells was performed with excess unlabeled oligonucleotides of the p53-binding sequence, and the nuclear extract derived from Raji cells was used as standard protein

Figure 6 presents results from one of these clones. Western blot analysis showed that cells transfected with p21^{WAF1} had an increased level compared to the control cells (Inset). However, the overexpression of p21^{WAF1} did not affect levels of p16^{INK4a}, cyclin A, cyclin D1, cyclin E, Cdk2, Cdk4, or Cdk6 (data not shown). Furthermore, we analyzed the effect of overexpression of p21^{WAF1} on the cell growth. Each cell line was cultured for 7 days. The control cells were confluent by day 6. Cells overexpressing p21^{WAF1}, on the other hand, had increased in cell number by day 7, while the growth was slightly inhibited.

Evidence of p53-Independent Pathway of p21^{WAF1} Expression Induced by IL-1 in High-Passage Human Fibroblasts—In the present study, IL-1 induced p21^{WAF1} expression without changing the p53 level in the WI38 cells, as presented in Fig. 1. These results confirmed our previous findings (*43*). However, many studies have shown that increased levels of p53 are required for the accumulation of p21^{WAF1}, while there are also reports that the expression of p21^{WAF1} does not require p53 (*6, 7, 12, 14–17, 20, 21, 24–26*). To determine the mechanisms responsible for the accumulation of p21^{WAF1} transcripts by IL-1, transcriptional run-on assays were performed (Fig. 7). p21^{WAF1} was constitutively transcribed in untreated high-passage WI38 cells; exposure of the cells to IL-1 for 2 h increased the transcriptional rate of p21^{WAF1} by 13-fold. Furthermore, the transcriptional activity of the p53 in these cells was assayed using a reporter plasmid (POST2) which has two copies of p53-responsive element upstream of the thymidine kinase (TK) promoter linked to the CAT reporter gene. IL-1–treatment of WI38 cells transfected with the reporter gene did not affect the CAT activity as compared to basal levels of CAT (Fig. 8). In contrast, co-transfection with the wild-type p53 expression vector markedly enhanced the CAT activity.

To determine the capability of p53 to bind a sequence-specific motif, gel shift assays were carried out with an oligonucleotide coding for the p53-responsive element from the human p21^{WAF1} promoter (Fig. 9). The DNA-binding activity was detected in untreated high-passage WI38 cells and was competed with excess unlabeled oligonucleotide. However, IL-1 did not affect the binding activity in these cells.

DISCUSSION

Cell-cycle progression is dependent upon extracellular growth-controlling signals including cytokines; cytokines act as stimulators or inhibitors of cell-proliferation by inducing the expression of various proteins that are required for the execution of cell-cycle processes (*3, 30, 31*). *In vitro*, human fibroblasts undergo a limited number of population doublings; growth and division of these cells generate a metabolically active but non-dividing population of senescent cells; this process has a genetic program (*17, 46, 50, 51*). Enhanced expression of p21^{WAF1} has been reported in senescent human diploid fibroblasts, suggesting that p21^{WAF1} expression may contribute to lower activity of senescing primary fibroblasts (*17, 46*). IL-1 causes growth arrest in the G1 phase of the cell-cycle in human tumor cells (*39, 52*). On the other hand, IL-1 activates cell proliferation (*29–31*). Our preliminary report showed that IL-1 induces p21^{WAF1} expression in human WI38 embryonic lung fibroblasts (*43*). In the present study, we have investigated further the mechanisms for induction of p21^{WAF1} and the effects of IL-1 on the cell-cycle in high-passage fibroblasts. IL-1 induced the expression of p21^{WAF1} both at the RNA and protein levels in high-passage WI38 fibroblasts but not in early-passage cells. However, neither an increased level nor

an enhanced function of p53 was seen in the high-passage cells, nor did IL-1 cause growth arrest.

Prior studies have found that addition of serum to starved diploid fibroblasts resulted in a rapid increase in the $p21^{WAF1}$ level, with a decrease at around the G1/S transition; levels of $p21^{WAF1}$ were below the levels before addition of serum (*17*, *60*). Furthermore, $p21^{WAF1}$ can be induced as an immediate early gene and is also expressed when quiescent fibroblasts and T lymphocytes are stimulated to proliferate by mitogenic signals; this expression is transient and these events also occur independently of functional p53 (*13*, *17*, *25*, *26*, *60*, *63–65*). Moreover, IL-1 is known to induce expression of immediate early genes (*36–39*). In the present study, however, treatment of high-passage WI38 cells with IL-1 markedly increased the $p21^{WAF1}$ level after 3 h of exposure, and the level remained higher than the baseline over the 24 h of analysis. Moreover, IL-1 did not stimulate proliferation of these cells. These results suggest that $p21^{WAF1}$ is induced by IL-1 through mechanisms different from those for growth factors and plays a role distinct from those of the immediate early genes.

In our study, no effects of IL-1 on the progression of cell-cycle were observed in high-passage cells. The Cdk inhibitors function by inhibiting Cdk-mediated phosphorylation of the Rb (*66*). Furthermore, previous studies have shown that Rb hypophosphorylation is required for the G1-arrest by IL-1 (*39*). However, IL-1 did not affect the status of Rb in high-passage WI38 fibroblasts. Further studies found that IL-1 did not affect the Cdk2, Cdk4, or Cdk6 kinase activity in these cells. On the other hand, if positive regulators of the cell-cycle such as Cdks or cyclins are induced by IL-1, they may overcome the otherwise growth inhibitory effects of $p21^{WAF1}$ (*67*, *68*). Cyclin D1 is known to reduce growth inhibition by TGF-β, which activates another Cdk inhibitor, $p27^{Kip1}$ (*69*); and cyclin D1 induces transcriptional activation of $p21^{WAF1}$ without altering cell-cycle progression through p53-independent mechanisms (*70*, *71*). To determine whether this was the situation in IL-1–treated high-passage fibroblasts, we analyzed levels of Cdk2, Cdk4, cyclin E, cyclin D1, and cyclin A or E associated with Cdk2. IL-1 showed no significant effects on expression levels of these positive regulators in these cells as compared to control cells treated with serum alone (data not shown). The further possibility that $p21^{WAF1}$ cannot bind to Cdk complexes efficiently was negated by our finding that IL-1 increased the level of $p21^{WAF1}$ coprecipitated with anti-Cdk2, Cdk4, or Cdk6 antibody. The observed discrepancy between $p21^{WAF1}$ binding and lack of inhibition of Cdk activities suggests that a Cdk inhibitor(s) other than $p21^{WAF1}$ may play an important role in the cell-cycle of these cells.

TNF is a cytokine that is cytotoxic or cytostatic to various malignant cells (*40*). While TNF and IL-1 are distinct cytokines and act through different receptors, they share many biological activities (*40*, *41*). A recent study has reported that $p21^{WAF1}$ was not involved in mediating cell-cycle arrest by TNF in ME180 cervical cancer cells (*52*). Moreover, there are reports that $p21^{WAF1}$ does not always act as a cell-cycle inhibitor; constitutively increased levels of $p21^{WAF1}$ have been observed in human brain tumors (*72*). In acute myelogenous leukemia, no correlation between the levels of $p21^{WAF1}$ and the kinase activity of the $p21^{WAF1}$ complexes has been reported (*73*). To determine whether $p21^{WAF1}$ plays a critical role in the growth of these WI38 cells, cells were stably transfected with a $p21^{WAF1}$ expression vector. Overexpression of $p21^{WAF1}$ in high-passage cells slightly inhibited the cell growth; the growth curve of $p21^{WAF1}$-expressing high-passage WI38 cells was similar to that of control cells. Taken together, our results suggest that $p21^{WAF1}$ induced by IL-1 does not act as a Cdk inhibitor in these cells, but they do not exclude the possibility that Cdk2 form an active complex with $p21^{WAF1}$ in high-passage cells.

The mechanisms responsible for the lack of effect of IL-1 on the cell-cycle are unknown. The Cip/Kip family of inhibitors bind to and inhibit a broad range of kinases (*74*). The Cip/Kip family share significant homology in their terminal region, suggesting that they may inhibit cyclin/Cdk activity in the same manner. A portion of the conserved region of $p21^{WAF1}$ (amino acids 46–78), which is homologous to similar regions in the Cip/Kip family, can bind to Cdk2. In the present study, IL-1 downregulated levels of $p27^{Kip1}$, a member of the Cip/Kip family, in high-passage WI38 cells. $p27^{Kip1}$ regulates Cdk2 activity during the transition from the G1 phase to the S phase; overexpression of $p27^{Kip1}$ blocks cell-cycle progression in G1 phase, and antisense vectors targeted to $p27^{Kip1}$ mRNA increase the fraction of cells in the S phase (*74*, *75*). $p27^{Kip1}$ also accumulates in quiescent human fibroblasts (*64*, *76*). Furthermore, it has been suggested that the increased expression of the $p16^{INK4a}$ may contribute to arresting the growth of senescent cells (*49*). However, IL-1 did not affect the levels of $p16^{INK4a}$ in the high-passage WI38 cells (data not shown). The Cdk2 structure consists of an N-terminal lobe that is rich in β-sheet, and a larger, C-terminal lobe that is mostly α-helical, with the catalytic cleft in between the two lobes (*77*). From crystal structural analysis, the N-terminal lobe and catalytic cleft of Cdk2 have been shown to bind the β-hairpin, β-strand, and 3_{10} helix of $p27^{Kip1}$, and the binding of $p27^{Kip1}$ to cyclin/Cdk causes conformational changes in the Cdk2 molecule (*78*). However, the site(s) on Cdk2 that are involved in $p21^{WAF1}$ binding have not been identified, while the amino-terminal half of Cdk2 is known to be important for $p21^{WAF1}$ binding (*79*). On the other hand, it has been shown that $p21^{WAF1}$ contains two copies of the cyclin-binding motifs (amino acids 17–24 and 146–164) (*80*), whereas $p27^{Kip1}$ contains only one cyclin-binding domain (*81*). Moreover, $p21^{WAF1}$ is frequently induced in cells entering the cycle from a quiescent state, whereas $p27^{Kip1}$ levels are generally high in quiescent cells but fall prior to their entry into S-phase (*65*). These studies suggest that differences may exist among members of this family in their binding affinity for specific Cdks. Unlike that of $p21^{WAF1}$, the expression of $p27^{Kip1}$ and $p16^{INK4a}$, which regulate the phosphorylation status of Rb (*82*, *83*), does not appear to be under p53 control. The regulation of members of the Cki family is quite varied in cells. $p21^{WAF1}$ may not play an important role in the cell growth in high-passage WI38 fibroblasts, and other Ckis including $p27^{Kip1}$ may be critical for the regulation of the cell-cycle in these cells.

The present study showed that the transcriptional rate of $p21^{WAF1}$ was increased in response to IL-1 in the WI38 cells. However, no increased levels of p53 were observed in these high-passage cells. A prior study has reported that binding and transcriptional activities of p53 were increased in high-passage cells but in the absence of increased p53 protein (*50*). Recently, two additional members of the p53

family, p73 and p51 have been also identified; both proteins can activate promoters through the p53-binding sequences (*84*). Therefore, we examined both DNA-binding activity *in vitro* and the transcriptional activity of p53 *in vivo* in high-passage cells. IL-1 did not induce the transcription of a CAT reporter gene containing p53 consensus-binding sequences. Furthermore, our DNA mobility shift assays demonstrated that IL-1 did not activate the DNA binding to p53 consensus sequences of nuclear extracts in high-passage WI38 cells. Therefore, our results suggest that increased levels of p21^{WAF1} mRNA in IL-1–treated cells are, at least in part, due to transcriptional activation that is independent of p53, probably p51, and p73. Recently, pathways of transcriptional activation of p21^{WAF1} that do not require p53 have been reported (*21*, *27*, *45*, *70*, *85*, *86*). Furthermore, the stage of differentiation or development may relate to the difference in the mechanisms of cell-cycle regulation between fibroblasts derived from mature tissues and immature tissues. The expression of p21^{WAF1} with differentiation or development is independent of p53 (*20*, *21*). Another study using embryonic fibroblasts derived from p21^{WAF1}-deficient mice has shown that DNA-damage–induced G1 arrest is only partially dependent upon p21^{WAF1} (*53*). Studies have also reported that the cell-cycle arrest caused by p21^{WAF1} was counteracted by SV40 large T antigen through binding to Rb (*62*, *87*). The expression of SV40 large T antigen has been shown to reduce levels of p21^{WAF1} (*88*). To determine whether our high-passage WI38 cells have endogenous SV40 T antigen, we performed Western blot analysis using the antibody against the large T antigen. However, the WI38 cells did not express the SV40 T antigen (data not shown). The mechanism(s) of transcriptional activation of p21^{WAF1} by IL-1 is unknown. IL-1 is also known to activate transcriptional factors (*40*). Further studies are required to identify the pathway independent of p53 for transcriptional activation of p21^{WAF1} by IL-1.

In summary, we have shown that IL-1 induced p21^{WAF1} in high-passage human embryonic fibroblasts but not in early-passage cells. However, the biological role of the p21^{WAF1} induced by IL-1 in these cells is unknown. In cancer cells, the constitutive expression of B-myb was shown to bypass the effect of p21^{WAF1}, while the mechanisms involved remain unclear, and the expression of c-myc was found to override the expression and the function of p21^{WAF1} (*89–91*). However, IL-1 did not affect levels of c-myc mRNA in these cells (data not shown). A recent study has reported that TNF induces a non-functional p21^{WAF1} that migrates faster than the usual p21^{WAF1} (*92*). Moreover, p21^{WAF1}-associated kinases have been reported to exist in both active and inactive states (*93*). We electrophoresed the proteins from these WI38 cells treated with IL-1 through 15% SDS–polyacrylamide gels; no faster-migrating band of p21^{WAF1} was observed (data not shown). There is also a report indicating that p21^{WAF1} is not required for senescence of Li-Fraumeni cells (*51*), emphasizing that the senescent program can involve more than one effecting pathway. Thus, extremely complex and multistep mechanisms are involved in the regulation of the cell-cycle. Identification and characterization of additional genes encoding growth inhibitors that are upregulated in senescent cells should provide a better understanding of the "aging program."

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