The G\textsubscript{1}-phase Growth-arresting Action of Interleukin-1 Is Independent of p53 and p21/WAF1 Function*

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Interleukin-1 (IL-1) causes G\textsubscript{1}-phase growth arrest of A375-C6 human melanoma cells by hypophosphorylation of the retinoblastoma susceptibility protein Rb. Because p53 and p21/WAF1 proteins are key components of growth arrest pathways involving Rb hypophosphorylation, we tested the functional role of these two proteins in IL-1 action. Exposure to IL-1 caused induction of both p53 and p21/WAF1 proteins. However, inhibition of p53 function by the K1 mutant of SV40-T antigen or by m175 (Arg to His) dominant-negative mutant of p53 did not result in abrogation of IL-1 action, suggesting that p53 function is not required for growth arrest by IL-1. Studies aimed at testing the role of p21/WAF1 in IL-1 action indicated that IL-1 induced p21/WAF1 expression independently of the p53 status of the cells. However, inhibition of p21/WAF1 expression resulted in only a marginal rescue from the growth-arresting action of IL-1. These findings imply that despite their induction, neither wild-type p53 nor p21 can fully account for the growth arrest by IL-1. Thus, a p53- and p21-independent pathway(s) mediates IL-1 action.

Inducer-dependent and cell type-specific gene programs result in regulation of cell cycle progression in response to exogenous stimuli. G\textsubscript{1}-phase genes are key regulators of the cell cycle; they determine entry into, or exit from, the cell cycle. Two key proteins that regulate entry from the G\textsubscript{1} phase to the S phase and thereby serve a checkpoint control function are p53 and the retinoblastoma susceptibility protein Rb (1–5). These two proteins function to cause G\textsubscript{1}-phase growth arrest in response to exogenous insults in diverse cell types (1–5).

The tumor suppressor gene p53 is a central mediator of cell cycle growth arrest and apoptosis (reviewed in Refs 6–8). The p53 protein encodes a transcription factor that functions as a transcriptional activator or repressor, depending upon the promoter context (9–12). A number of p53-responsive genes, such as GADD45, MDM2, muscle creatine kinase gene, epidermal growth factor receptor, thrombospondin, bax, cyclin G, the insulin-like growth factor-binding protein 3, and p21/WAF1/CIP1 are implicated in growth inhibition and apoptotic cell death (13–16). Both transcription activation-dependent and -independent mechanisms play a role in the growth inhibitory action of p53 (17–20). Wild-type p53 protein levels generally increase in response to genotoxic stress and either mediate cell cycle arrest or lead to apoptosis (21–23). In a number of different types of cancers, p53 is the most commonly mutated tumor suppressor gene (24, 25). Lack of p53 expression or function is associated with an increased risk of tumor development (26–29). Consistent with this observation, ectopic overexpression of p53 in normal or transformed cells causes cell cycle arrest and/or apoptosis (3).

One of the p53-inducible genes, p21/WAF1/CIP1, inhibits the growth of human tumor cell lines and normal diploid fibroblasts in the G\textsubscript{1} phase (16, 30). p21/WAF1-inducible growth arrest involves direct inhibition of cyclin-dependent kinase activity that then results in hypophosphorylation of Rb protein and accumulation of the cells in the G\textsubscript{1} phase of the cell cycle (31–34). Additionally, p21/WAF1 protein can also cause growth inhibition via the Rb-independent pathway(s) (1). On the other hand, some stimuli induce p21/WAF1 independently of p53 and cause G\textsubscript{1} growth arrest (35). Moreover, a number of studies have shown p21/WAF1 expression in p53 knock-out mice or in cells containing mutant forms of p53, thereby reinforcing the view that p21/WAF1 can be regulated by a p53-independent mechanism (35–37).

IL-1,\textsuperscript{1} a polypeptide hormone, is a major immunoregulatory cytokine secreted predominantly by activated macrophages and monocytes (38–40). IL-1 plays diverse biological roles in the host system, and the growth regulatory effects of IL-1 are generally nongenotoxic and cell-type-dependent (38–44). IL-1 inhibits cell proliferation by causing programmed growth arrest in human melanoma cells A375-C6 (41, 43). IL-1-inducible growth arrest in these cells is receptor-mediated and dictated by immediate early gene expression events (41–44). IL-1 induces a number of immediate early genes in human melanoma cells. These include gro-\textalpha, gro-\beta, IRG-9, c-jun, NAK-1, MAD3, and Egr-1 (41–44). Egr-1 protein blocks the growth arrest action of IL-1 (40), but the function of the other immediate early genes is not known. IL-1 action is mediated by hypophosphorylation of Rb protein that leads to growth arrest in the G\textsubscript{1} phase of the cell cycle (44). However, the early events leading to Rb hypophosphorylation by IL-1 have not been defined. In studies aimed at identifying the early events linked to IL-1-inducible growth arrest, we found that IL-1 causes induction of p53 and p21/WAF1 in A375-C6 cells. Because these proteins are linked to G\textsubscript{1} phase growth arrest in many different cell types, we sought to determine whether the induction of p53 and p21/WAF1 contributes to the growth-arresting action of IL-1. Data presented here suggest that growth arrest by IL-1 occurs by a p53- or p21/WAF1-independent mechanism.

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\textsuperscript{1}The abbreviations used are: IL-1, interleukin 1; CAT, chloramphenicol acetyltransferase.
MATERIALS AND METHODS

Cell Culture and Cytokine—Human melanoma A375-C6 cells were grown in RPMI 1640 medium containing 10% fetal bovine serum. Human recombinant IL-1β (specific activity 1.8 x 10^7 units/mg) was a gift from Craig Reynolds, Biological Response Modifiers Program, National Cancer Institute (Frederick, MD).

Plasmid Constructs and Transfection of Cells—The p21/WAF1 antisense and deletion mutant constructs (16), the p53-m175 expression construct that encodes mutant p53 protein, and reporter constructs PG13-CAT or MG15-CAT that contain intact or mutant binding sites for p53 (45) were kindly provided by Bert Vogelstein (Johns Hopkins University of Medicine, Baltimore, MD).

Western Blot (immunoblot) Analysis—Whole-cell protein extracts were prepared and analyzed by Western blot analysis as described previously (44, 47). The anti-p21/WAF1 polyclonal antibody that recognizes the peptide corresponding to amino acids 146–164 at the carboxyl terminus of human p21/WAF1 and anti-p53 monoclonal antibody DO-1 that recognizes an amino-terminal epitope (residues 37–45) of human p53 were from SantaCruz Biotechnology, Inc. (Santa Cruz, CA). Equal loading of the protein was verified by reprobing the blots with an anti-β-actin antibody purchased from Sigma.

Northern (RNA) Analysis—Isolation of total RNA and Northern analysis were performed as described previously (47, 48). The blots were probed with a full-length cDNA probe for human p53 (45) or p21/WAF1 (35) and subsequently with cDNA for glyceraldehyde-3-phosphate dehydrogenase to ascertain equal loading of RNA.

Assay for Growth Arrest—Subconfluent cultures of A375-C6 cells were subjected to [3H]thymidine incorporation studies, as described

FIG. 1. Nuclear transcriptionally active p53 is induced by IL-1 in A375-C6 cells. a, A375-C6 cells were untreated (UT) or treated with IL-1 for various intervals of time (in h) as indicated. Whole-cell protein extracts were prepared and subjected to Western blot analysis for p53 or β-actin. b, A375-C6 cells were left untreated (UT) or treated with IL-1 for 5 h and then subjected to immunocytochemistry for p53. Brown dianobenzidine stain indicates p53 expression; the bluish green color is indicative of methyl green counterstaining. c, A375-C6 cells were transfected with PG13-CAT or MG15-CAT, and expression of CAT activity was assayed by thin layer chromatography.
Previously (41–44), to quantify the growth arrest action of IL-1.

**Immunocytochemistry**—A375-C6 cells were left untreated or exposed to IL-1 for 5 h, and then immunocytochemistry was performed with anti-p53 antibody DO-1 as described previously (49).

**RESULTS**

**IL-1 Induces Nuclear p53 Expression in A375-C6 Melanoma Cells**—Because IL-1 causes G1 phase growth arrest, we tested the possibility that this action of IL-1 is linked to induction of p53. The cells were exposed to IL-1 for different time intervals, and then whole-cell protein extracts were examined by Western blot analysis for p53 expression. As seen in Fig. 1a, IL-1 induced the expression of p53 protein in 2 h, and the levels stayed elevated for about 5 h.

Previous studies have shown that p53 protein in the inactive state is localized in the cytoplasm but in the active state it translocates to the nucleus, where it may function to regulate gene transcription (50–52). To localize p53 protein, we used immunocytochemistry on A375-C6 cells that were untreated or treated with IL-1 for 5 h. Studies with the p53-specific antibody DO-1 indicated that 40 to 60% of cells in untreated A375-C6 cultures showed weak staining for p53 protein (Fig. 1b, left panel). As noted previously (49), in the untreated cells that expressed p53, the protein was detected primarily in the nucleus. When treated with IL-1, more than 98% of the cells in culture showed strong staining for p53 protein in the nucleus (Fig. 1b, right panel). These results indicated that in untreated A375-C6 cells, p53 protein was primarily localized in the nucleus and that the cultures showed an increase in nuclear expression of p53 upon treatment with IL-1.

The role of p53 in tumor cell growth inhibition has been related to its ability to act as a transcription factor (3, 21). Because p53 is wild type (49) and located in the nucleus of A375-C6 cells (Ref. 49; this study), it can potentially function as a transcription factor. To determine whether p53 in A375-C6 cells is transcriptionally active, we transiently transfected A375-C6 cells with PG13-CAT (45), which contains 13 p53-response elements placed upstream of the CAT cDNA, and measured CAT activity in the transfectants. For a control, we transfected the cells with MG15-CAT (45), which contains 15 mutant copies of the p53 response element placed upstream of CAT cDNA. As seen in Fig. 1c, A375-C6 cells showed strong expression of the p53-responsive PG13-CAT construct relative to the mutant construct MG15-CAT. These data suggest that A375-C6 cells express transcriptionally active p53 protein.

**p21 Is Induced by IL-1 at the RNA and Protein Level**—p21/WAF1 expression is induced transcriptionally by p53 via two p53 binding sites in the gene promoter (53). Moreover, the ability of p53 to cause growth arrest in the G1 phase of the cell cycle is mediated by p21/WAF1 protein (16). Because IL-1 was found to induce p53 in A375-C6 cells, we tested whether IL-1 also induced the induction of p21/WAF1 in these cells. A375-C6 cells were left untreated or treated with IL-1 for various intervals of time, and Northern blot analysis was performed by using a full-length cDNA probe for human p21/WAF1. As seen in Fig. 2a, IL-1 treatment for 2 h resulted in an approximately 3-fold increase in the mRNA level of p21/WAF1 when compared with basal levels in untreated cells. Elevated levels of p21/WAF1 mRNA were seen up for 8 h of exposure to IL-1. Next, we determined whether IL-1 caused an increase in the expression of p21/WAF1 protein. A375-C6 cells were left untreated or treated with IL-1 for various intervals of time, and whole-cell protein extracts prepared from the cells were subjected to Western blot analysis for p21/WAF1. As shown in Fig. 2b, IL-1 caused an induction of p21/WAF1 protein. A 3-fold increase in p21/WAF1 protein levels was noted at 2 h of IL-1 treatment, and elevated levels were maintained for at least 4 h. These findings suggested that IL-1 causes induction of p21/WAF1 at the mRNA and protein level.

**Overexpression of the K1 Mutant of SV40 T Antigen Blocks Transcriptional Activation Function of p53 but Does Not Protect the Cells from IL-1-inducible Growth Inhibition**—We have shown that A375-C6 cells contain wild-type, transcriptionally active p53. To inactivate p53 protein and then study its role in IL-1 action, we utilized either wild-type or mutant forms of SV40 T antigen (cited in Ref. 44). Wild-type T antigen can bind and sequester both p53 and Rb, whereas the K1 mutant (which contains a point mutation in the large T protein at amino acid 107, converting a Glu to Lys) binds to p53 but not to Rb. We reasoned that if the function of wild-type p53 was necessary for IL-1-inducible growth inhibition, overexpression of the wild-type or mutant forms of T antigen should sequester p53 and thereby protect the cells from IL-1 action. In preparing to address this question, we first ascertained that the wild-type and K1 forms of T antigen abrogated the ability of p53 in A375-C6 cells to cause transcriptional activation. These studies used the previously described A375-C6 transfectants that stably expressed either empty vector (for control) or the SV40 wild-type or mutant protein (44). The cell lines were tested for transcriptionally active p53 by transient transfection with either PG13-CAT or MG13-CAT. As seen in Fig. 3a, wild-type T antigen or K1 mutant severely reduced p53-specific transactivation in transfected cell lines. These data suggested that both the wild-type and mutant forms of T antigen can abrogate p53 function.

To determine the role of p53 in the growth-arresting action of IL-1, parent A375-C6 cells or the cell lines stably transfected with vector, SV40 T antigen or the K1 mutant were treated with IL-1 for 48 or 72 h, and the effect on growth was examined by [3H]thymidine incorporation studies. As seen in Fig. 3b, A375-C6 parent cells or vector transfectants that did not express SV40 protein showed about 50 or 80% growth inhibition.
in response to IL-1 exposure at 48 or 72 h, respectively. Transfecteds expressing wild-type T antigen were relatively resistant to growth arrest by IL-1 (Fig. 3b). By contrast, transfecteds expressing the K1 mutant showed growth arrest in response to IL-1 with kinetics similar to those seen in the parent cells or in cells transfected with vector (Fig. 3b). Because the K1 mutant, which inhibits p53 transactivation function, failed to abrogate the growth arrest action of IL-1, p53 is not a critical component in the action of IL-1.

Dominant-negative p53 Does Not Protect Cells from the Growth Arrest Action of IL-1—Another approach to determine whether p53 protein in A375-C6 cells is functionally important in the growth-arresting action of IL-1 involved the use of a p53 dominant-negative mutant m175 that can inhibit the ability of wild-type p53 to function as a transcriptional activator (45, 54). This mutant lacks transactivation potential and can form protein oligomers with wild-type p53 by binding amino acids at the carboxyl terminus and thereby inhibiting the function of wild-type p53 (45, 54). To determine the effect of ectopically overexpressed mutant p53 protein on the transactivation function of endogenous wild-type p53, A375-C6 cells were transfected with the p53-m175 expression construct pCMV-m175 or with vector for a control. Pools were made from about 100 individual stably transfected clones, and m175 or vector were tested for controls for their ability to compete with endogenous wild-type p53 for transactivation of the reporter construct PG13-CAT by CAT assays. As described previously (54), transfecteds expressing mutant p53 protein showed much lower CAT activity from PG13-CAT than transfecteds containing the vector (Fig. 4a).

These results confirmed that the mutant form of p53 protein can inhibit transcriptional activation by endogenous wild-type p53 in A375-C6 cells. Next, the transfecteds expressing p53 mutant protein or vector were used to determine whether p53 was responsible for IL-1-inducible growth arrest. These cells were exposed to IL-1 for 24, 48, or 72 h, and growth inhibition was studied by [3H]thymidine incorporation assays. As seen in Fig. 4b, p53-m175 did not rescue the cells from the growth-arresting action of IL-1. Together the observations from the above experiments that used the K1 mutant of SV40 T antigen or the p53 m175 dominant-negative mutant to abrogate wild-type p53 function indicated that the growth-arresting action of IL-1 is mediated by a p53-independent pathway.

IL-1 Induces p21/WAF1 Independently of p53 Function and Inhibition of p21/WAF1 Expression Only Marginally Rescues the Cells from IL-1 Action—To determine the role of p53 in IL-1-mediated induction of p21/WAF1, we used A375-C6/SV40 T.L1 or A375-C6/SV40 K1.L1 cell lines transfected with the construct for wild-type SV40 T antigen or the K1 mutant of T antigen described previously (44). The cell lines were previously shown to express the respective SV40 proteins (44). Because the wild-type T antigen or the K1 mutant form complexes with p53 protein and sequesters it, thereby inactivating it, p53 can be expected to be functionally inhibited in these cell lines. Lack of functional p53 in these cells was directly demonstrated by the inability to transactivate the PG13-CAT reporter construct (see Fig. 3a). The cell lines were treated with IL-1 for various time intervals, and then IL-1 induction of p21/WAF1 was examined by Western blot analysis. As seen in Fig. 5, p21/WAF1 was induced by IL-1 in both the cell lines at a magnitude similar to that seen in parent A375-C6 cells (compare with Fig. 2). Thus, p21/WAF1 can be induced by IL-1 in cells where p53 is functionally inactivated by sequestration by T antigen or its K1 mutant. These findings imply that IL-1 induces p21/WAF1 independently of p53.

Next, to determine whether p21/WAF1 induction was necessary for IL-1-inducible growth arrest, we transfected A375-C6 cells with the expression construct pC-WAF1-antisense that expresses p21/WAF1 cDNA in the antisense orientation or with pC-WAF1-deletion mutant that expresses a nonfunctional deletion mutant of p21/WAF1. Stable clones were selected, and at least three clones were tested for p21/WAF1 expression in the presence or absence of IL-1 by Western blot analysis or for sensitivity to IL-1 by [3H]thymidine incorporation studies. Whole-cell protein extracts were prepared from transfected
cells or, for control, parent A375-C6 cells that were untreated or treated with IL-1 for 3 h. As seen in Fig. 6a, the parent cells showed induction of p21/WAF1 upon IL-1 treatment. Cells transfected with the p21/WAF1 antisense construct did not show detectable expression of p21/WAF1 even after treatment with IL-1 (Fig. 6a). By contrast, as compared with the parent cells, the induction of p21/WAF1 by IL-1 was unaltered in cells transfected with pC-WAF1 deletion mutant (Fig. 6a). These findings suggested that p21/WAF1 antisense but not the p21/WAF1 deletion mutant inhibited the induction of p21/WAF1 by IL-1.

We then exposed the transfectants, which expressed either the antisense or deletion mutant of p21/WAF1, to IL-1 for various amounts of time and performed [3H]thymidine incorporation experiments to determine their susceptibility to the IL-1. Parent A375-C6 cultures or clones expressing p21/WAF1 deletion mutant showed about 20 or 45% growth inhibition in response to the IL-1 in 24 or 48 h, respectively. Clones expressing the p21/WAF1 antisense construct showed about 15 or 35% growth inhibition in response to IL-1 at 24 or 48 h, respectively (Fig. 6b). Thus, the commitment of A375-C6 melanoma cells to growth arrest after IL-1 treatment is only marginally dependent on p21/WAF1 gene induction. These observations on the marginal rescue of the cells expressing p21/WAF1 antisense construct from IL-1 action were reproducible in multiple experiments. These findings suggest that antisense p21/WAF1 does not totally protect A375-C6 cells against IL-1 action and that a p21/WAF1-independent pathway(s) mediates IL-1 action.

**DISCUSSION**

Cytokines such as IL-1 or tumor necrosis factor α provide a natural defense mechanism against tumor cells (40, 55, 56). IL-1 causes growth arrest in the G1 phase of the cell cycle in human melanoma cells A375-C6 (44) and in breast carcinoma cells MCF-7 (57). Our previous studies have shown that Rb hypophosphorylation is required for the G1-phase growth-ar-
cell line ME-180 was recently shown to be independent of p21 function (58). Perhaps other cyclin-dependent kinase inhibitors such as p27 or p15 that regulate the phosphorylation status of Rb (59, 60) may be recruited by IL-1 for its growth arrest action.

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