Gene Expression, Vol. 7, pp. 377–385, 1999
Printed in the USA.

1052-2166/99 $10.00 + .00

Functional Role of p21 During the Cellular Response to Stress

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A wide range of stress stimuli, including oxidants, genotoxins, metabolic deficiencies, and irradiation, have been shown to induce expression of the cyclin-dependent kinase inhibitor p21. Among the best characterized mediators of p21 induction by stress is the tumor suppressor gene p53, which acts as a transcriptional activator to enhance the expression of the p21 gene. However, many other mechanisms involving transcriptional and posttranscriptional events have been found to participate in the elevation of p21 levels by stressful agents. The significance of the stress-mediated elevation in p21 expression is not fully understood, but it is clear that alterations in p21 expression impact on the ability of the cell to survive the insult. Although a large number of reports have demonstrated correlations between the expression of p21 and cellular outcome, this review will focus only on those reports where the role of p21 in a given stress paradigm has been investigated directly, through use of different strategies to manipulate p21 expression followed by assessment of the consequences of altered p21 expression on cell survival. The majority of such studies have revealed that p21 exerts a protective function against stress, and this property appears to rely, at least in part, on the ability of p21 to suppress cell proliferation. A few exceptions to this universal protective influence of p21 have also been observed and will be discussed.

Cip1 Waf1 Sdi1 Cyclin-dependent kinase inhibitor Stress response Genotoxic stress p53 Growth arrest Apoptosis Gene induction

FOLLOWING exposure to stressful stimuli, mammalian cells activate a number of response mechanisms associated with the induction of numerous so-called stress-response genes. The particular genes induced are largely dependent on the nature of the insult, but there is considerable overlap in the cell's response to diverse stimuli. Although the precise function of many of the stress-inducible genes is yet to be determined, they are believed to ultimately contribute to the determination of the cell's fate, which can range from proliferation to growth arrest, differentiation, senescence, and cell death. Among those genes whose expression is highly upregulated during stress is the cyclin-dependent kinase inhibitor p21, which plays an important role in regulating cell growth (12,24,40).

An important component of the stress response is a temporary or permanent alteration in the cell division cycle. Cell cycle regulation in eukaryotes involves the sequential activation of cyclin-dependent kinases (cdks) that drive cell cycle progression through phosphorylation of key regulatory proteins [for review, see (39)]. Cdk activity is modulated positively in various ways, most notably through binding of regulatory cyclins, and negatively, through mechanisms including association with cdk inhibitors. Cdk inhibitors fall into two families: one comprised of p16 (Ink4a), p15 (Ink4b), p18 (Ink4c), and p19 (Ink4d), and another consisting of p21 (Waf1, Sdi1, Cip1), p27 (Kip1), and p57 (Kip2) [for reviews, see (35,41)]. Although certain stresses have been shown to affect the expression of several of these cdk inhibitors, only p21 has been found to be highly induced by a wide spectrum of stress conditions (19). Given its ability to act as a universal inhibitor of cdks (55), it is believed to directly participate in the growth ar-

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rest that ensues following exposure to harmful stimuli. Whether this growth arrest is important to assure the cell's survival or, alternatively, contributes to the onset of apoptosis has been the topic of much study.

This review will focus on two aspects of p21 induction by stress. First, we will provide a brief overview of the mechanisms that serve to upregulate p21 expression during stress. Second, we will address the importance of p21 expression in determining survival during the cellular response to stress.

MECHANISMS REGULATING STRESS-INDUCIBLE p21 EXPRESSION

p21 was independently isolated, at around the same time, by several different groups utilizing different strategies. One of these strategies sought the identification of inhibitory proteins associated with cdks (24). Another group demonstrated that p21 was upregulated by the tumor suppressor p53, itself a stress-activated transcription factor (12). A third team identified p21 based on its enhanced expression in senescent cells (40). Since these initial studies, p21 expression has been shown to be induced by a large variety of stresses through complex mechanisms involving both transcriptional and posttranscriptional processes. Although a detailed analysis of such processes is not the focus of this review, we will provide a brief summary of these regulatory mechanisms.

Transcriptional Regulation of p21 Expression During Stress

Transcriptional control of p21 expression has been the focus of numerous studies and it has been the topic of a recent review to which the reader is referred (17). Although many transcription factors have been implicated in the regulation of p21 expression, we will limit our discussion to those shown to play a role in stress-induced p21 expression.

p53. The p53 tumor suppressor protein is a transcription factor responsible for regulating the expression of several genes. Comparison of the rat, mouse, and human p21 promoter sequences reveals the conservation of at least two p53 binding sites, and the minimum requirement of one intact p53 binding site for transcriptional activation (13). Studies using various systems of p53 deficiency have demonstrated that, whereas the majority of inducers of p21 expression do not require the presence of functional p53, in most instances, p53 does contribute to the magnitude of p21 expression seen. An exception to this theme is p21 induction following exposure to DNA-damaging agents, which relies heavily on the presence of functional p53 (11,19,36). Indeed, in the case of ionizing radiation, p53 is absolutely required for the induction of p21 (11). Hence, the p53-dependent growth arrest of cells exposed to DNA-damaging agents and other stresses is widely believed to be mediated via p21.

SP1. The p21 promoter sequence contains six SP1 binding sites in its proximal region. Under different stress conditions, different sites are required for binding of each member of this multigene family (SP1, SP2, SP3, SP4), often in association with other transcription factors such as with Smad proteins following exposure to TGF-β, or with p300/CBP after elevations in intracellular Ca^{2+} [(4); for review, see (17)].

Other transcription factors found to regulate transcriptional activation of p21 expression by stress-related conditions include AP2, required for TPA-induced transcriptional activation of the p21 gene (57), CEBPβ in response to antioxidants (9), and STATs following exposure to IFNγ, thrombopoetin, or IL-6 (3,26,37).

Posttranscriptional Regulation of p21 Expression During Stress: p21 mRNA Stabilization

Enhanced p21 expression by stressful stimuli can also be mediated through alterations in the stability of the p21 mRNA. The 2.1-kb p21 mRNA contains a short coding region (492 b) which is followed by a long 3′ untranslated region (UTR) containing sequences characteristic of short-lived mRNAs (7). These include AU-rich stretches and three copies of the pentamer AUUUA (44). Although the half-life of the p21 mRNA in normal growing cells is about 1 h, exposure to various stresses, such as γ-irradiation or treatment with either TPA, TNF-α, diethylmaleate, or okadaic acid increases its half-life to greater than 4 h (1,15,43,58). Recently we reported that short wavelength ultraviolet light (UVC) also induces p21 expression through a posttranscriptional mechanism involving stabilization of the p21 mRNA (22). Intriguingly, however, this induction is also dependent on the presence of functional p53. Although the p53 protein has been shown to have the ability to bind RNA and may thus regulate mRNA stability directly, it is more likely that p53 regulates the expression or activity of one or more proteins involved in mRNA stabilization, for example, a certain RNase, an RNA binding protein, or some other protein(s) involved in regulating their expression and/or activity. The identity of the putative protein(s) that regulate p21 mRNA stability remains unknown, but its function appears to involve tyrosine phosphorylation, because inhibition of tyrosine phosphatase activity us-

ing vanadate restored UVC-mediated inducibility in p53-deficient cells (22).

Very recently, Joseph et al. (27) reported that HuD, a member of the *elav*-like family of RNA binding proteins, binds in vitro in a site-specific manner with high affinity to the p21 mRNA. HuD is a neuronal-specific protein whose interaction with short-lived RNAs is associated with enhanced stability. In collaborative efforts, our group and Dr. Furneaux's have recently obtained evidence that the p21 mRNA binds another member of the *elav*-like protein family, HuR, whose expression is ubiquitous (34). Further, the formation of the [p21 transcript–HuR] complex was found to be inducible by UVC irradiation. The significance and details of this process are the subject of active investigation in our laboratory. Evidence that HuR binding to the p21 mRNA is critical for its stabilization following UVC treatment has been obtained using an experimental approach involving constitutive expression of antisense HuR to lower endogenous HuR levels. Cells expressing lower HuR levels exhibited reduced binding to the p21 mRNA, diminished p21 mRNA inducibility after UVC treatment, and a shorter p21 mRNA half-life, indicating that HuR is required for p21 induction by UVC and that p21 mRNA stabilization is regulated by HuR (Wang et al., unpublished results). This study has also provided evidence that HuR may likewise participate in regulating p21 expression by other stress agents.

Translational and Posttranslational Regulation of p21 Expression

p21 expression is also subject to regulation through direct alterations in the steady-state levels of the p21 protein. The early work of Bae et al. (2) revealed a disparity among certain cell lines in that they expressed equally high p21 mRNA following γ-irradiation, but very different levels of the p21 protein. Although the precise regulatory mechanisms contributing to these differences were not fully understood at that time, they indicated that the expression of p21 was likely to be regulated by alterations in protein stability (2). In this regard, a recent report by Fukuchi et al. (16) may shed light on the possible mechanisms mediating the increased stability of p21: exposure of ML-1 cells to the DNA-damaging agent etoposide was found to prevent ubiquitination of p21, thereby allowing p21 to accumulate in the cell. Ubiquitin/proteasome-dependent regulation of p21 levels had also been previously demonstrated (5).

Recent reports by Timchenko et al. (47,48) have provided evidence for additional regulatory mechanisms leading to an increase in the half-life of the p21 protein. C/EBPα, a member of the C/EBP family of transcription factors whose expression is restricted to adipose and liver tissues, regulates the expression of p21 through direct protein–protein interaction, as shown both in cultured cells and in intact animals. Association between p21 and C/EBPα results in the inhibition of p21 proteolysis and renders the p21 protein more stable. This regulatory process contributes in part to the ability of C/EBPα to function as a general regulator of cell proliferation during liver development and specifically after a stress stimulus such as partial hepatectomy. Whether other members of the C/EBP family might exert a similar function remains to be determined.

Finally, p21 can be specifically cleaved by a CPP32-like caspase, yielding a truncated protein that no longer inhibits cdk2 activity. As discussed below, this form of posttranslational regulation may greatly impact on apoptosis of both normal and transformed human cells, as a mutant p21 protein that cannot be cleaved partly prevents apoptotic cell death (30a,56a).

ROLE OF p21 WITHIN THE STRESS RESPONSE

As the number of reports showing that p21 expression is highly regulated by stress has increased, so has the interest in understanding the role of p21 during the stress response. Initially, two opposing views emerged regarding the function of p21 during the stress response: one argued that p21 played a role in the implementation of apoptosis while the other proposed that p21 serves a protective function, preventing apoptosis. The first argument was based largely on the fact that p21 was a p53-regulated gene. Because p53 has been tightly linked to apoptosis, it was logical to assume that being a downstream effector of p53, p21 was part of that process. The second view hypothesizes that p21 expression is important for the growth arrest that occurs following stress, a process that is necessary to allow the cell time to repair damage to its DNA or other molecules before it is passed on to daughter cells. According to this model, high levels of p21 in cells undergoing apoptosis may reflect a "failed attempt" on the part of the cell to mount a survival response. Currently, the majority of published reports supports a general protective function of p21 against cellular stress, but there are instances in which p21 has no measurable benefit for the cell and still others in which elevations in p21 are detrimental for the cell.

Protective Function of p21 During Cellular Stress

One of the first studies addressing the influence of p21 on cellular outcome was performed in our labora-

tory utilizing the cyclopentenone prostaglandin A_2 (PGA_2) as the stress agent (18). A survey of cell types revealed that treatment of most cells with PGA_2, including MCF7 breast carcinoma lines, led to growth inhibition associated with increased p21 expression, and little or no cytotoxicity. However, a small subset of cell lines, such as RKO colorectal carcinoma cells, neither growth arrested nor elevated the expression of p21 in response to PGA_2 and the cells succumbed to treatment with the drug (18). This correlation led us to postulate that p21 may directly participate in determining the cellular outcome. Indeed, we were able to demonstrate that inhibition of p21 expression in MCF7 cells (through stable transfection with a vector expressing an antisense p21 transcript) prevented the PGA_2-mediated growth arrest of MCF7 cells and, importantly, sensitized them to the cytotoxic influence of PGA_2 treatment (18). Conversely, ectopic expression of p21 in RKO cells, achieved through infection with an adenovirus carrying a p21 cDNA, led to a marked protection of RKO cells against PGA_2 cytotoxicity (20).

Another study assessing the role of p21 on cellular outcome employed SH-SY5Y neuroblastoma cells. Here, treatment of cells with aphidicolin and nerve growth factor leads to neuronal differentiation and an elevation in p21 expression. Use of antisense oligonucleotides to block p21 expression in SH-SY5Y cells undergoing differentiation resulted in apoptotic cell death, again indicating that p21 was required for cell survival under these conditions (42). Likewise, reduced p21 expression, achieved through adenovirus-mediated delivery of an antisense p21 transcript, markedly sensitized glioblastoma cells to apoptosis triggered by chemotherapeutic agents (42a).

Waldman et al. (51) also reported that p21 exerts a protective function against cell death following treatment with various antitumor agents. Using HCT116 colorectal cancer cells, they developed isogenic lines carrying deletions of both of p21 alleles through homologous recombination. Comparing the sensitivity of HCT116 cultures with differential p21 status towards various chemotherapeutic agents, they found that cells lacking p21 showed enhanced cytotoxicity in response to adriamycin, γ-irradiation, etoposide, or camptothecin treatments. In addition, while cells with normal p21 status underwent growth arrest and retained normal DNA content (2N and 4N) following treatment with the DNA-damaging agents, cells lacking p21 showed an accumulation of cells with 8N and 16N DNA content, as they underwent additional replication processes (S phases) without mitoses (M phases). This uncoupling between the S and M phases led to polyploidy and other chromosomal aberrations, and contributed, the authors argued, to the heightened susceptibility of the p21-deficient cells to apoptosis (51). In support of this hypothesis, these authors also showed, in a separate study, that p21-deficient cells exhibit a defect in DNA repair following exposure to various DNA-damaging agents (38). The authors suggest that defects in p21 expression could explain the higher sensitivity of some tumors to chemotherapeutic drugs: many cancers lack functional p53, and p53 is required, to varying extents, for the induction of p21 after exposure to DNA-damaging agents and other chemotherapeutic drugs (50). Thus, inability to fully induce p21 may render the cells more susceptible to selective killing. A further corollary of this model is that noncancer cells, exhibiting normal p53 status, would survive the treatment.

Using the same cell system (p21-deficient and -proficient HCT116 cells) Wouters et al. (54) reported that the preferential sensitivity to γ-irradiation exhibited by p21-null cells was also observed in vivo in xenografts irradiated in situ: tumors derived from normal HCT116 cells were more refractory to killing by ionizing radiation, whereas tumors derived from p21-deficient HCT116 cells were much more sensitive to radiation as determined both by clonogenic survival and by regrowth of tumors following treatment. This increased sensitivity to killing by γ-irradiation was further accentuated in mice doubly null for the p21 and atm genes, and was accompanied by a massive apoptotic response, as reported by Wang and coworkers (53).

The protective influence of p21 during stress has also been studied in other systems. As shown by Sheikh et al. (46), short wavelength ultraviolet light (UVC) was found to be much less cytotoxic for DLD1 colorectal cells where p21 expression was ectopically induced by a tetracycline-inducible system than for parental counterparts expressing low p21 levels. As proposed by the authors, p21 may modulate the process of DNA repair that is required following UV-induced DNA damage, thus contributing to the improvement in cell survival seen in p21-expressing cells. In a related study, we compared wild-type mouse embryo fibroblasts with counterparts either lacking p53 or p21, for their ability to survive following UVC irradiation (22). We also observed a markedly reduced survival rate in populations deficient for either p21 or p53 function. In this stress system, p53 is required for increasing p21 expression, although this elevation in p21 levels is not through changes in the transcription of the p21 gene, but rather through stabilization of the p21 mRNA, as indicated above. Treatment of cells with sodium vanadate restores UVC-mediated p21 expression in p53-

deficient cells and this is associated with enhanced survival.

Based on these intriguing correlations between p53, p21, and cell death, we sought to directly address whether p21 might participate in p53-triggered death or survival by examining the effect of ectopic p21 expression in melanoma cells undergoing apoptosis by p53 overexpression. Endogenous p21 expression was very low and unchanged in melanoma cells undergoing apoptosis after p53 overexpression (21). However, adenovirus-mediated delivery of p21 led to a dramatic reduction in p53-induced apoptosis of melanoma cells. Given that the p53-triggered responses (apoptotic cell death on one hand, and enhanced survival through growth inhibition on the other) appear to be mutually exclusive, at least in part, this example strongly suggests that p21 may serve to preferentially shift the p53-mediated response to one of survival.

Finally, there are hundreds of examples where p21 induction by various agents correlates with enhanced cell survival, although the role of p21 in each of these instances was not assessed directly. Similarly, high levels of p21 expression in tumors has been reported to correlate with heightened resistance to chemotherapy and poor prognosis (56).

Lack of Detectable Consequences of p21 on Cellular Outcome During Stress

In one among a handful of reported examples where p21 fails to modulate cell survival after stress, Erdhardt and Pittman (14) observed that the presence of p21 did not alter the survival of PC12 rat pheochromocytoma cells subjected to serum withdrawal-induced apoptosis. Here, the survival of parental PC12 cells was indistinguishable from that of PC12 cells engineered to express p21 through a tetracycline-regulated system. That is, p21 expression neither enhanced nor reduced serum withdrawal-induced cytotoxicity. However, it is important to note that this is a rather unique situation in that serum deprivation is one of the few stresses that actually reduces rather than elevates p21 expression. In another report, Sheikh et al. (45) showed that overexpression of p21 under control of a tetracycline-regulated promoter failed to protect DLD1 colorectal carcinoma cells towards adriamycin treatment. Several other studies have also reported no appreciable effect of p21 overexpresssion on cell survival (28,29), but its influence on subsequent stressful treatments was not assessed in those studies.

Other examples where p21 fails to exhibit a detectable influence on cellular outcome were found as part of our laboratory's long-term interest in understanding the cell's response to oxidative stress. We had observed that exposure of HeLa cells to hydrogen peroxide (H_2O_2) led to a mild elevation in p21 expression that was subject to rather narrow time- and dose-dependent limits. As H_2O_2 leads to apoptosis in these cells (52), we investigated whether p21 overexpression would influence the cytotoxicity seen. To this end, HeLa cells that were either mock-infected, infected with an "empty" virus (Ad.null), or infected with an adenovirus expressing p21 (Ad.p21) were treated with H_2O_2 and apoptosis was assessed at various times following treatment. As shown in Fig. 1, H_2O_2 treatment was equitoxic for each of the infection groups. In other experiments, we have confirmed this inability of p21 to protect against H_2O_2-mediated apoptosis in another cell model in which p21 is overexpressed in a tetracycline-regulated fashion (Huang et al., unpublished results).

Detrimental Influence of p21 Expression During Stress

Several groups have also reported examples that implicate p21 in enhancing cell death. In one of the earliest examples, Sheikh and coworkers reported that ectopic overexpression of p21 in MCF7 or T47D cells resulted in apoptotic cell death, in association

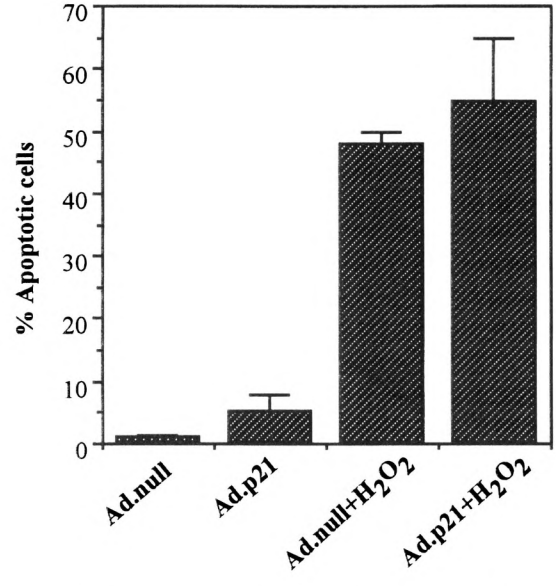

FIG. 1. Effect of ectopic p21 expression on H_2O_2-triggered apoptotic death of HeLa cells. HeLa cells were infected with either Ad.null (an adenovirus carrying no insert) or Ad.p21 (an adenovirus expressing a p21 cDNA), with a virus dose of 100 plaque-forming units (pfu)/cell (sufficient to infect greater than 90% of the cell population). Twenty-four hours following infection, cell cultures were treated with 600 μM H_2O_2 and apoptotic cells were scored 24 h later. Apoptotic cells were visualized after staining with the DNA dye DAPI, using standard methods (52). Values are the means ± SE obtained from three separate experiments.

with growth arrest and giant cell formation (45). Kondo et al. described a similar observation in U87 glioma cells, where transfection with a p21 expression vector led to their apoptotic death (30). However, the same authors found that overexpression of p21 in another glioma cell line, GB-1, did not directly lead to cell death, although it did sensitize them to cisplatin-induced apoptosis (30).

Our own group, while exploring the influence of p21 expression during stress, has also found a limited number of situations where p21 expression is detrimental for the cell. Infection of Rat1 cells with Ad.p21 was extremely cytotoxic, leading to a very rapid and dramatic demise of all infected cells (Fig. 2). Ad.null-infected cells failed to exhibit this toxic response, indicating that p21 was responsible for the observed toxicity.

SUMMARY AND CONCLUDING REMARKS

In conclusion, although p21 expression during the stress response is tightly regulated at multiple levels (transcriptional, posttranscriptional, and posttranslational), its influence on the cellular outcome is still not fully understood. Here, we have reviewed studies where p21's effect on the outcome of stressed cells has been directly assessed following manipulations to elevate or reduce its expression. The presence of p21 was found to be protective in most instances, as reported in numerous studies using a variety of cell lines of different origins subjected to a wide range of toxic treatments. Several examples exist, however, where alterations in p21 expression have no impact on cellular outcome. We suspect that the relative scarcity of such published examples is partly due to the fact that negative results are less likely to come to publication. Still, a few studies have shown that p21 expression can be detrimental for cell survival. Investigations yielding these results, however, lacked important additional (or "complementary") experiments; for example, in instances where p21 expression exacerbates cytotoxicity by stress agents, it has not been shown that inhibiting p21 expression restores protection towards the given stress agent. Nevertheless, the importance of p21 during the cellular response to stress can vary significantly from one cell type to the next, and from one stress condition to another. Better understanding of the precise functions of this growth regulatory gene will be necessary to understand the basis for such differential effects. In addition, it is important to keep in mind that the relative importance of p21 expression in a given stress condition is also dependent on what other stress response mechanisms (pro-survival or pro-apoptotic) have been mounted via independent regulatory pathways. As depicted in the model (Fig. 3), we share the emerging view that p21 generally exerts a protective influence in instances of cellular stress. We still do not understand, however, how p21 performs this protective function. It is possible that its beneficial role is directly related to p21's ability to induce growth arrest through its inhibitory effect on cyclin-dependent kinases (6,10). Elevated p21 levels can halt cell cycle progression, providing additional time for the cell to repair the damage inflicted by the stressful agent. Although this is a logical and plausible hypothesis, there has been no direct experimental demonstration to support its validity. We believe that only a systematic and detailed analysis of the p21 protein may begin to shed light on the nature of its protective function. A fruitful approach may be, for example, the sort of analysis that has been carried out over the past few years on the p53 protein, involving dissection of its various domains to discern the nature of its apoptotic, transcriptional, and growth-inhibitory functions, and begin to understand their relationships and interconections (23,32). Likewise, dissection and separate analysis of p21's domains involved in interaction with cdks, PCNA, etc. (8,31,33,49), may perhaps provide an answer to whether or not its growth-inhibitory function can be separated from its protec-

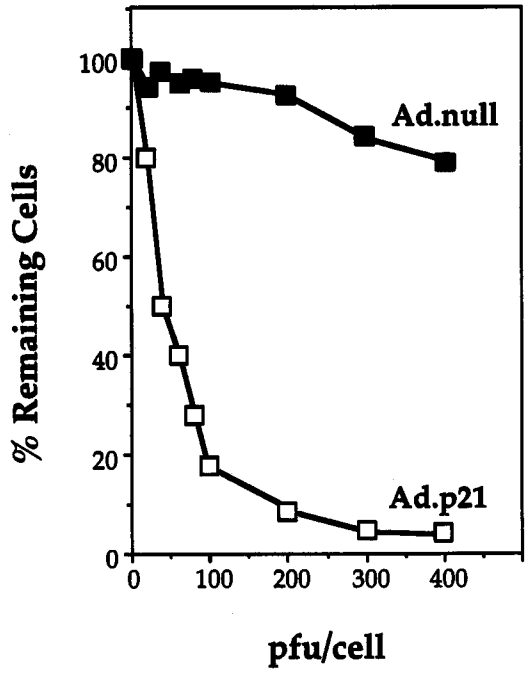

FIG. 2. Cytotoxic influence of p21 overexpression on Rat1 fibroblasts. Rat1 cells growing on 96-well plates were infected with various amounts of either Ad.null or Ad.p21 adenoviruses (described in the legend of Fig. 1). Cell survival was scored 18 h after infection using crystal violet as described (20). Results are represented as the percentage of cells remaining relative to uninfected cells in each treatment group.

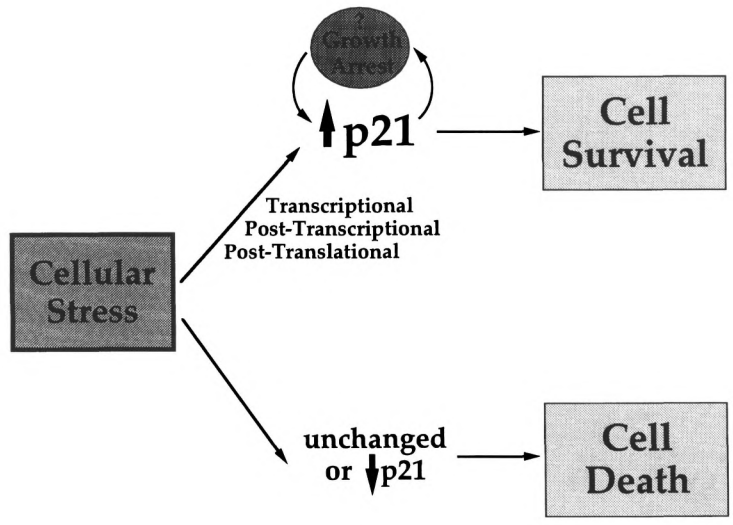

FIG. 3. Schematic representation of the influence of p21 expression on the outcome of the stressed cell. Model depicts the predominant view on the influence of p21 on cell survival following exposure to stressful treatments. Stress-triggered induction of p21 expression (through transcriptional, posttranscriptional, or posttranslational mechanisms) generally leads to an enhancement in cell survival, whereas unchanged or reduced p21 levels (or perhaps even an insufficient elevation in p21 expression) fail to protect cells against stress-induced cell death. That growth arrest is critically required for p21's implementation of the survival response is a possibility favored by many investigators, but it remains to be experimentally demonstrated.

tive capacity. Such an approach has already provided clues about the nature of p21's protective influence. As demonstrated by Levkau et al. (30a), p21-mediated inhibition of cdk2 activity was critical in order for p21 to protect towards death of human endothelial cells: cleavage of p21 abrogated this inhibition, led to a marked elevation of cdk2 activity, and cells underwent apoptosis. Preventing this inhibition, through use of either a dominant-negative cdk2 or a mutant p21 that could not be cleaved, partly restored survival. These results strongly support the notion that p21 exerts its protection through inhibition of cellular proliferation.

Finally, the demonstration that p21 influences the cellular outcome joins a growing body of evidence that alterations in cell cycle regulation profoundly impact the response of stressed cells (25) and could have direct clinical relevance for the treatment of cancer, as radiation therapy and various chemotherapeutics can clearly induce p21 expression. A comprehensive understanding of the influence of p21 (or other cell cycle regulators) on the cellular outcome could lead to the design of experimental approaches aimed at heightening the sensitivity of cancer cells to a given therapeutic agent. In light of the protective role of p21 described here, a reduction in p21 expression, through interference with its transcriptional or posttranscriptional regulators, antisense RNA approaches, or other means, would result in the enhanced demise and elimination of cancer cells.

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