Induction of Transcriptionally Active Jun Proteins Regulates Drug-induced Senescence*

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The drug hydroxyurea (HU) is used for cancer therapy and treatment of sickle cell anemia. It inhibits cell cycle progression by blocking DNA synthesis and drives cells to undergo apoptosis or enter senescence. We demonstrate here that HU induces the expression of two AP-1 proteins, c-Jun and JunB, which exert antagonistic effects on the cell cycle. Moreover, the induction of c-Jun is observed following treatment with two other drugs that inhibit the cell cycle in S phase, aphidicolin and camptothecin. The induction of c-Jun, which promotes cell cycle progression, up-regulates expression of cyclin D after exposure of cells to HU. Deficiency in c-Jun prevents elevation of cyclin D expression and extends entrance into HU-induced senescence but also renders cells more resistant to HU-dependent apoptosis. The induction of c-Jun is independent of JNK activity, and additionally, of c-Jun autoregulatory activity but is inhibited upon inhibition of protein kinase C activity. Therefore, we suggest that c-Jun activity prevents drug-induced senescence. Conversely, the JunB target gene, tumor suppressor p16^INK4a, a cyclin-dependent kinase inhibitor essential for the induction of drug-induced senescence, is also up-regulated by HU in a JunB-dependent manner. Constitutive expression of JunB up-regulates p16^INK4a and increases the sensitivity of mouse fibroblasts to drug-induced senescence. Thus, we suggest that in contrast to c-Jun, JunB drives cells to enter HU-dependent senescence. The effect of HU treatment, which regulates the intricate web of AP-1 transcription, depends on the balance between c-Jun and JunB activities.

Replicative senescence, into which most cells enter after a defined number of divisions, is characterized by the loss of replicative and proliferative abilities, whereas cellular viability is retained (1). Initially observed in cultured cells, it was recently demonstrated to be a physiologically relevant phenomenon against oncogenic stress (3, 5). Cellular senescence, together with apoptosis, prevents cancer development and stops oncogenic transformation (2). Interestingly, both cases, the tumor suppressor p53 and the CDK2 inhibitors p21cip1 and p16^INK4a are essential regulators of senescence (2, 5). Thus, it is believed that both the p53 and the retinoblastoma (Rb) pathways are essential for its induction. As p53 activity is up-regulated by DNA damage, it is not surprising to find its involvement in the process. However, the ability of retinoblastoma to enhance both oncogenic and genotoxic-induced senescence is more surprising and requires further investigation. Recent studies have demonstrated that SUV39h1-dependent methylation of histone H3 lysine 9, which occurs in Ras-induced senescence and adriamycin-induced senescence, is Rb-dependent (6, 7). In addition, deletion of p16^INK4a reduces the efficiency of drug treatment against lymphomas without changing the apoptotic response of the tumor cells (2). Furthermore, in agreement with a model suggesting that Rb activities play a central role in the induction of senescence, it is hypophosphorylated in senescent cells (8), and its phosphorylation by cyclin/CDK complexes disturbs its association with repressive co-factors such as histone deacetylase (9).

One drug that induces senescence is hydroxyurea (HU), synthesized more than a hundred years ago. Its negative effect on leukocyte proliferation was first observed in 1928 (10). For the last 30 years, it has been used to treat several neoplastic diseases, including carcinoma of the head and neck and chronic myeloid leukemia (reviewed in Ref. 11). It is also used for the treatment of non-neoplastic diseases, including sickle cell anemia, as it induces fetal hemoglobin synthesis (reviewed in Ref. 12). Biochemically, HU exerts its antiproliferative activity mainly by inhibiting ribonucleotide reductase activity, which blocks DNA synthesis and repair (13). In addition, exposure to HU also leads to DNA damage, mainly double strand breaks that induce the expression and activity of proteins, including the p53 tumor suppressor (14–17), involved in the response to DNA damage. Thus, HU treatment can lead either to arrest of proliferation or to cell death, depending on the dose and length of exposure.

The AP-1 proteins are highly responsive to environmental stresses. c-Jun level and activity are activated by numerous

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stresses that activate the c-Jun amino-terminal kinase (JNK) (reviewed in Ref. 18). The regulation of c-Jun expression includes positive autoregulation of c-Jun expression (18). JunB, on the other hand, is not positively regulated by JNK (19). Despite this fact, both are induced by genotoxic stresses, such as UV radiation (20, 21). The biological significance of this co-induction is obscure as the proteins seem to antagonize under conditions of co-expression but, in the absence of c-Jun expression, JunB can compensate for some of the activities of c-Jun in development (reviewed in Ref. 18). c-Jun deficiency leads to retarded proliferation (22), whereas JunB deficiency does not, and its overexpression results in inhibition of fibroblast proliferation (23). In fact, JunB elicits tissue-specific effects in the hematopoietic compartment, which assign it to be a tumor suppressor. A myeloproliferative disorder, which resembles early human chronic myeloid leukemia, appears in mice lacking JunB expression due to increased numbers of long term hematopoietic stem cells due, at least in part, to increased proliferation (24, 25). In vitro skin reconstitution models also demonstrate the differences in the ability of c-Jun or JunB fibroblasts to support the proliferation of keratinocytes (26). In addition, c-Jun is a regulator of cell survival, leading cells to apoptosis upon induction by genotoxic and exitotoxic stresses or starvation to survival factors, whereas JunB does not enhance cell death (18). At least some of the antagonistic biological effects elicited by the two Jun members stem from opposing effects on the Rb pathway. Although c-Jun attenuates this pathway by the induction of cyclin D (27, 28), JunB is a positive regulator of p16INK4a, an inhibitor of the CDK4/6/cyclin D activity (23). Concordantly with the role of c-Jun in the inhibition of senescence, c-Jun-deficient mouse embryo fibroblasts enter premature senescence after two passages in culture, whereas the levels of JunB, and consequently, of p16INK4a are increased as mouse fibroblasts divide toward senescence (23, 29, 30).

c-Jun and c-fos were reported to be regulated by HU (31); however, the biological significance of their induction is not totally clear. We tested the expression and activity of AP-1 proteins in cells exposed to HU and determined their role in inducing senescence. c-Jun and JunB were found to be the AP-1 proteins most potently induced by HU. c-Jun is induced independently of JNK and of the autoregulatory activities of c-Jun. Nevertheless, the c-Jun target gene cyclin D is induced by HU in a c-Jun-dependent manner, but p16INK4a is also up-regulated by HU. Most important, we show that mouse fibroblasts deficient in c-Jun expression are far more susceptible to senescence than c-Jun-expressing cells and are more resistant to the apoptotic effects of HU. We thus suggest that c-Jun may modulate the outcome of chemotherapeutic treatment.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatment—Mouse fibroblasts, HeLa, and transformed human embryonic kidney (HEK293) cells were grown at 37 °C in an atmosphere of 5% CO2 in high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The cells were treated with hydroxyurea (Calbiochem) at a concentration of 5 mM or aphidicolin, camptothecin (Sigma), and 12-O-tetradecanoylphorbol-13-acetate (TPA) at a concentration of 1–5 μM, and calphostin C (Calbiochem) was added at a concentration of 100 nM.

Western Blot Analysis—Whole cell extracts were prepared and analyzed for the detection of c-Jun. Nuclear extracts were analyzed for JunB. Proteins were separated by 10% PAGE and transferred to Immobilon-P transfer membrane (Millipore). c-Jun and JunB levels were detected using H-79 and N-17 antibodies, respectively (Santa Cruz Biotechnology, Santa Cruz, CA). Actin was detected using anti-actin (l-19) antibody, and p16 was detected using 50.1, F-12, or M156 antibodies (Santa Cruz Biotechnology). Phosphorylated myristoylated alanine-rich protein kinase C substrate (MARCKS) was identified using antibodies against p-MARCKS (Ser-159/163) sc-12971 (Santa Cruz Biotechnology).

RNA Analysis—Total RNA was purified from cells using the EZ-RNA kit (Biological Industries). RNA was separated on 0.9% agarose gels and blotted onto Nytran SuPerCharge (Schleicher & Schuell). Probes were radiolabeled with [32P]dCTP using a random prime labeling kit (Stratagene). Primers used for real-time PCR analysis of cyclin D1, junB, and p16INK4a expression will be provided upon request. An MX 3000p instrument (Stratagene) was used for the analysis.

Cell Cycle Assays—Control and treated cells were pulse-labeled with BrdUrd for 30 min before harvesting. Determination of cell cycle was performed at different times following treatment by staining the cells with 5 μg/ml propidium iodide (Sigma) and fluorescein isothiocyanate-conjugated anti-BrdUrd antibody (BD Biosciences). Both adherent and floating cells were collected for analysis.

Immunocomplex Kinase Assay—JNK immunoprecipitation and in vitro kinase assays using glutathione S-transferase-tagged amino-terminal c-Jun fragment (1–79) as a substrate were performed as described previously (32). Anti-JNK1 antibody G151-333.8 (Pharmingen) was used for immunoprecipitation.

Determination of Cellular Senescence—Cellular senescence was determined by analysis of β-galactosidase activity as described previously (33). At least 400 cells were counted at each point.

Plasmids—Retroviral vector expressing JunB ShRNA was previously described (34). Human junB cDNA was cloned into pBabe to generate JunB retroviral expression vector.

RESULTS

AP-1 Proteins Are Induced by Hydroxyurea—The drug HU inhibits DNA synthesis and damages DNA. To comprehensively study the effect of HU on the expression of AP-1 proteins that are responsive to genotoxic stress, we employed Northern analysis to screen the expression of different AP-1 members after exposure of mouse fibroblasts to 5 mM HU. As depicted in Fig. 1A, exposure to HU slightly increased junD and c-fos expression but strongly induced junB and c-jun expression. Therefore, we decided to focus on these AP-1 members. Mouse fibroblasts were exposed to HU, and the kinetics of c-jun mRNA induction was examined by Northern analysis. Unlike the induction of c-jun mRNA after exposure to UV radiation or serum stimulation, which is very rapid, its induction by HU starts only 3–4 h after treatment and persists as long as the cells are exposed to the drug (Fig. 1B). Elevation of c-Jun protein
levels after treatment with HU is observed in mouse fibroblasts (Fig. 1D), as well as in HeLa and HEK293 cells (data not shown). JunB mRNA induction is also relatively late in HeLa cells exposed to HU (Fig. 1C), and JunB protein is elevated by HU in mouse fibroblasts as expected (Fig. 1D). These results demonstrate that both c-Jun and JunB are induced by HU and that the induction is not cell line-specific.

We next tested whether c-Jun is induced by any type of forced arrest of proliferation. The induction of c-jun mRNA after exposure of mouse fibroblasts to 5 μM aphidicolin, another inhibitor of DNA synthesis, suggested that the induction of c-Jun is not dependent on effects specific to HU (Fig. 2A). In fact, as low as 0.5 μM aphidicolin induced c-Jun protein expression, and like HU, also increased JunB expression (Fig. 2B). To test whether c-Jun induction is a common phenomenon to all drugs that arrest proliferation at the S phase, we also treated mouse fibroblasts with 5 μM camptothecin, another chemotherapeutic drug that interferes with the normal association/dissociation of topoisomerase I with the DNA. As depicted in Fig. 2C, camptothecin also induces c-Jun expression, leading to the notion that forced arrest of DNA synthesis accompanied by DNA damage leads to c-Jun induction.

The induction of c-Jun by the above agents may reflect a response to DNA damage or G1/S-dependent expression of c-Jun. To test this point, the effect of growth arrest elicited by a physiological condition, which does not damage DNA, on c-Jun expression was examined. Mouse fibroblasts were plated at different densities ranging from low to full confluence and harvested 24 h later. c-Jun levels were inversely correlated with cell confluence and were dramatically reduced at high confluence (Fig. 3A). This result is expected as JNK and p38 activities are reduced in confluent cultures (35, 36). To further scrutinize the relations between the HU-dependent induction of c-Jun and changes in cell cycle, we compared both parameters in treated cells. As damage by HU is replication-dependent, we used cells plated at high but not saturation density with HU and tested the effects on cell cycle and c-Jun expression. As depicted in Fig. 3C, a higher portion of cells is arrested at G1 in the confluent culture, and the cells present at the S phase synthesize 2–10-fold less DNA than the sparse ones. c-Jun expression was inversely correlated to confluence as demonstrated above (Fig. 3, B and A). In addition, the minor change in cell cycle observed after treatment did not correlate to the kinetics of c-Jun induction. Although 80% of the cells were arrested at the G1/S phase as early as 12 h after treatment, c-Jun was induced 24 h after it despite the lack of change in cell cycle profile. Our results therefore support the notion that the induction of c-Jun by HU does not tightly correlate with increase in subpopulation in the G1/S phase of the cell cycle.

**c-Jun Inhibition Is Independent of the JNK Pathway but Depends on PKC Activity**—The JNK pathway is considered to be the major one responsible for c-Jun induction following exposure of cells to environmental stress. We therefore tested whether it is involved in c-Jun induction by HU. Initially, we used an immunocomplex kinase assay to test the ability of HU to activate JNK. Mouse fibroblasts were treated with HU and harvested at different time points, and the ability of immunoprecipitated JNK to phosphorylate bacterially produced c-Jun was determined. As depicted in Fig. 4A, the activation of JNK at various times preceding the induction of c-jun transcription or

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**FIGURE 1. c-Jun and JunB are induced by HU.** Mouse fibroblasts were treated with 5 mM HU and harvested at the indicated times (hours), and expression of the indicated AP-1 members (A) or c-jun only (B) was determined by Northern analysis. C, c-Jun mRNA levels were determined in HeLa cells exposed to HU and harvested at the indicated times. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. D,ac-Jun and JunB protein levels in mouse fibroblasts, which were treated for the indicated times (hours) with 5 mM HU, were determined by immunoblotting using c-Jun and JunB-specific antibodies. Nuclear extracts were prepared from mouse fibroblasts to determine JunB protein levels. Actin and lamin served as loading controls.

**FIGURE 2. c-jun is induced by aphidicolin and camptothecin.** A, mouse fibroblasts were treated with 5 μM aphidicolin for 12 h, and c-jun mRNA levels were determined by Northern analysis. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B, similar cells were treated with the indicated doses of aphidicolin and harvested 24 h later, and c-Jun and JunB protein levels were determined by immunoblotting. Lamin served as loading control. C, mouse fibroblasts were treated with 5 μM camptothecin for the indicated times, and c-Jun protein levels were determined by immunoblotting.
at 24 h after treatment, a time point at which \textit{c-jun} reaches peak levels, is marginal. By contrast, UV irradiation strongly activated JNK shortly after exposure, and high levels of activity were still observed 24 h after irradiation. To test whether the low JNK activation by HU accounts for the late \textit{c-Jun} induction by HU, we compared \textit{c-Jun} induction in \textit{JNK1}\textsuperscript{−/−} \textit{JNK2}\textsuperscript{−/−} double mutants after exposure to HU with that in \textit{JNK1}\textsuperscript{−/−} \textit{JNK2}\textsuperscript{−/−} mouse fibroblasts. The induction was comparable (Fig. 4B), proving that JNK phosphorylation is not essential for the efficient induction of \textit{c-Jun} by HU. Accumulation of \textit{c-Jun} \textit{63/73AA} and \textit{c-Jun} \textit{91/93AA} in mouse fibroblasts derived from knock-in mice after exposure to HU further supports this conclusion (data not shown). \textit{c-Jun} autoregulates its transcription (37). After exposure to environmental stresses, DNA binding and trans-activation of \textit{c-Jun} are dramatically increased, thus enabling it to enhance transcription of AP-1-regulated target genes, including \textit{c-jun} itself. To further determine whether \textit{c-Jun} plays any role in its mRNA induction following exposure to HU, we measured the levels of \textit{c-jun} mRNA in \textit{c-jun}\textsuperscript{−/−} fibroblasts before and after HU treatment. These cells express a fusion transcript containing truncated \textit{c-jun}, which lacks the sequences coding for the DNA binding domain, fused to the neomycin resistance gene, and its expression can therefore be monitored using a probe that identifies the 5′ of the molecule (38). The cells were treated with HU, and the expression of \textit{c-jun} mRNA at 12 and 24 h after treatment was determined by Northern analysis (Fig. 4C). Interestingly, \textit{c-jun} mRNA induction is detected following exposure to HU even in the absence of active \textit{c-Jun} protein. These results suggest not only that the JNK pathway is dispensable for \textit{c-jun} induction by HU but that even \textit{c-Jun} itself is not essential for the process.

As p38 and protein kinase C (PKC) are also known regulators of \textit{c-Jun} expression, it was of interest to examine their involvement in the HU-dependent induction of \textit{c-Jun}. Mouse fibroblasts were treated with HU in the presence or absence of specific inhibitors for the p38 and PKC kinases (SB203580 and calphostin C, respectively), and the induction of \textit{c-Jun} was measured. The addition of p38 inhibitor did not interfere with \textit{c-Jun} induction by HU despite negative effects on \textit{c-Jun} induction by UV, which served as a positive control for the inhibitor activity (data not shown). However, calphostin C, an inhibitor of the PKC kinases, significantly inhibited \textit{c-Jun} induction by HU (Fig. 4D). This result suggests that PKC kinases are involved in \textit{c-Jun} induction by HU; nevertheless, it is not known whether PKC activity is enhanced following exposure to HU. To test whether HU activates PKC, we examined the phosphorylation of the known PKC substrate, the 80-kDa MARCKS, which is a major \textit{in vivo} substrate of PKC (39, 40). Mouse fibroblasts were exposed to 5 mM HU, 30 J/m\textsuperscript{2} UV, or 1 μM TPA, and the level of MARCKS phosphorylation was determined by immunoblotting using specific phospho-Ser-159/163 antibodies. As depicted in Fig. 3E, TPA strongly induced the phosphorylation of MARCKS, as expected, UV did not increase it, and HU induced MARCKS phosphorylation, thus indicating that it activates PKC. These results suggest that HU induces PKC activity and that \textit{c-Jun} induction by HU is dependent on this activity.
Involvement of the ATR in c-Jun induction was also tested. c-Jun induction by HU was tested in the absence or presence of 5 mM caffeine, an inhibitor of ATR. Interestingly, for unknown reasons, caffeine reduced the basal level of c-Jun but did not inhibit its induction by HU, therefore suggesting that ATR is not involved in c-Jun induction by UV (supplemental Fig. 1).

\[ p16^{INK4a} \text{ and Cyclin D1 Are Induced by HU—} \]

HU induces the expression of c-Jun and JunB. However, it is not clear whether the HU-induced AP-1 proteins are active or transcriptionally aberrant. To test this point, the expression of the c-Jun target gene, cyclin D1 (27, 28), was measured in mouse fibroblasts treated with HU. As demonstrated previously (Fig. 1), c-jun mRNA is induced in HU-treated cells about 3 h after treatment (Fig. 5A), whereas cyclin D1 induction is delayed and is initially observed 12 h after treatment. Later, however, cyclin D1 is highly induced. This result suggests that at late times after treatment, cyclin D1 level correlates with c-Jun induction. To determine whether the increase in cyclin D1 levels are dependent on c-Jun, its expression was measured by real-time PCR analysis in c-jun\(^{-/-}\) and c-jun\(^{+/+}\) mouse fibroblasts treated with HU and harvested 12 and 24 h after treatment. As depicted in Fig. 5B, the expression of cyclin D1 is increased after exposure to HU only in cells expressing c-Jun and not in cells deficient in it, thus suggesting that cyclin D induction by HU is c-Jun-dependent. Interestingly, unlike exposure to HU, exposure of the cells to UV radiation, which is a very strong inducer of c-Jun, did not increase cyclin D1 expression despite stronger elevation in c-Jun levels (Fig. 5C and data not shown). These results suggest that the effects on cyclin D1 induction are more specific to HU.

In contrast to the growth-promoting activity of c-Jun, JunB up-regulates growth-inhibiting genes, including the tumor suppressor p16\(^{INK4a}\) (23). It was therefore of interest to examine the expression of p16\(^{INK4a}\) in cells treated with HU, which is, after all, an inhibitor of proliferation. Mouse fibroblasts and HeLa cells were treated with HU, and the expression of the p16\(^{INK4a}\) protein was measured by Western analysis. As depicted in Fig. 5, D and E, p16\(^{INK4a}\) is induced by HU in both cell lines. These results suggest that exposure to HU induces the expression of two genes possessing opposite effects on cell cycle progression through the induction of opposite effects on Rb activity.

\[ c-Jun \text{ Expression Inhibits Entrance into Senescence but Promotes Apoptosis—} \]

We next tested the biological consequences of c-Jun induction by HU. As HU induces senescence and apoptosis, we analyzed the effects of c-Jun on these processes in mouse fibroblasts. c-jun\(^{-/-}\) and c-jun\(^{+/+}\) mouse fibroblasts were treated with low doses of HU for 6 days and analyzed for senescence-associated \(\beta\)-galactosidase (SA-\(\beta\)-gal) activity, an established marker of senescence. Following treatment with HU, proliferation of both cell types was halted, and the cells assumed the flattened morphology characteristic of senescence. However, the morphological changes as well as SA-\(\beta\)-gal activity were more pronounced in c-Jun-deficient cells than in WT counterparts after HU treatment (Fig. 6A). This result indicates that cells lacking c-Jun expression are more sensitive to HU-induced senescence. Quantitative measurements of the
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A. 

- HU

- c-jun−/−

+ HU

B. 

Senescence %

Days after HU

0 1 2 3 4 5 6

44.9 20.3 18.2 27.2 4.4 3.1

C. 

Time: 0 8 24 60

BrdU incorporation

DNA content

c-jun+/+

c-jun−/−
process revealed that the percentage of senescence in cells deficient in c-Jun, in which cyclin D expression is not increased by HU, is higher than that of c-jun-expressing cells at every time point examined (Fig. 6B). The basal level of senescence in c-Jun-deficient cells is higher than in c-jun-expressing cells as described previously (29, 30); however, in both cases, it is quite marginal. Nevertheless, the HU-induced senescence in c-Jun-deficient cells was 30% higher than in the WT cells after 3 days. This difference in senescence further extended after 6 days; at that time, 44.9% of the c-Jun-deficient cells entered senescence, whereas only 20.3% of the WT cells did so. These differences are statistically significant ($p < 0.01$). Thus, our results suggest that c-Jun activity attenuates the entrance of HU-exposed cells to senescence.

As persistent c-Jun induction may also increase cell death (reviewed in Ref. 18), it was of interest to determine the effects of c-Jun expression on survival of HU-treated cells. c-Jun-deficient and c-jun-expressing mouse fibroblasts were treated with 5 mM HU, pulse-labeled with BrdUrd, and harvested at different times. Cell cycle distribution was determined by FACS analysis (Fig. 6C). In agreement with previous data, cells deficient in c-Jun are impaired in their proliferative capacity (22), and less BrdUrd labeling is therefore detected in comparison with the WT counterparts. In addition, these cells possess higher levels of spontaneous apoptosis (1.1%), which is reflected by the increased population containing sub-G1 DNA content, in comparison with the WT cells (0.1%). HU treatment dramatically reduced the rate of DNA synthesis in both cell lines as early as 8 h after treatment. However, the level of apoptosis in WT cells 60 h after treatment increased 166-fold, to 16.6%, in comparison with the minor increase observed in the c-Jun-deficient cells (a 6-fold increase to 7%). Thus, the induction of c-Jun by HU contributes to HU-dependent cell death. Similar results were also observed in c-Jun-expressing and -deficient mouse fibroblasts after exposure to aphidicolin (supplemental Fig. 2).

**JunB Enhances HU-induced Senescence**—The correlation of induction of JunB and p16INK4a raises the possibility that the induction of the last by HU is JunB-dependent. To explore the possibility, mouse fibroblasts were infected with retrovirus expressing ShRNA for JunB or not, selected, and shortly afterward exposed to HU for 12 h. Examination of JunB and p16INK4a expressions revealed that the ShRNA blocked JunB induction by HU. More importantly, it blocked p16INK4a induction by HU, thus proving that the induction is JunB-dependent (Fig. 7A). Interestingly, cell lines in which JunB was knocked down were relatively unstable, thus precluding the possibility of examining the biological effects of JunB deficiency on HU-induced senescence. Therefore, we infected the cells with retrovirus expressing JunB and selected them to generate cell pools expressing ectopic JunB. Examination of JunB and p16INK4a mRNA expression in these pools revealed a 3- and 6-fold increase, respectively (Fig. 7B). Concordantly, the morphology of cells expressing JunB changed, and they became bigger, flatter, and proliferated more slowly, as predicted from cells expressing high levels of p16INK4a (Fig. 7C and data not shown).

**Drug-induced senescence emerges as an important mechanism in the determination of the therapeutic efficacy of drugs in...**

![FIGURE 7. JunB regulates p16INK4a induction by HU and enhances HU-induced senescence. A, mouse fibroblasts expressing vector alone (Control) or ShRNA to JunB (JunB Sh) were exposed to HU for 12 h, and JunB and p16INK4a levels in the treated cells were determined by immunoblotting. B, control, JunB, and p16INK4a mRNA expression in cell lines of mouse fibroblasts infected with vector alone (Vec) or with JunB-expressing retrovirus (JunB). Black bars represent junB levels, and white bars represent p16INK4a levels. C, the same cell lines were exposed to 0.1 mM HU, and SA-β-gal activity was analyzed 4 days after exposure.](image-url)
c-Jun Inhibits HU-induced Senescence

cancer therapy (41). HU induces senescence-like changes in several cell lines including the erythroid progenitor cell line K562 and human diploid fibroblasts (42, 43). Here, we demonstrate that in addition to activation of factors directly involved in response to DNA damage, AP-1 proteins and their target genes are also regulated by HU expression. Previous reports demonstrated that c-Jun and Fos are induced in the erythroid progenitor cell line K562 following treatment with low doses of HU (31). We demonstrate here that this phenomenon occurs in most cell types examined and that JunB is also concomitantly and potently induced. Furthermore, two other drugs that interfere with the replication machinery, aphidicolin and camptothecin, also up-regulate c-Jun expression. The induction of c-Jun by forced arrest of DNA synthesis is possibly caused by the associated drug-induced DNA damage as arrest of replication due to confluence did not increase, but rather repressed, c-Jun expression, probably due to a reduction in the activities of INK and p38 in confluent cultures (35, 36). In addition, the kinetics of c-Jun induction differs in confluent cells from the kinetics of accumulation of cells in the G_1/S phase of the cell cycle after HU treatment. The relatively late induction of c-Jun in confluent cells may reflect the fact that the damage generated by agents interfering with the replication process is lower than in sparse ones as the cells do not replicate due to the high confluence. The induction of c-Jun by a DNA-damaging agent is thought to be mediated mainly by JNK activation. This raises the question as to the identity of the c-Jun inducer by HU, as we show here that elevated levels of c-jun mRNA are independent of both JNK and c-Jun. Instead, HU activates PKC, and its inactivation by a pharmacological inhibitor prevents the induction of c-Jun by HU. So far, activation of PKC by HU has been reported only in Saccharomyces cerevisiae (44). PKC is a family of serine/threonine kinases that are important in various biological processes. Under different conditions, the AP-1 proteins c-Jun, JunB, and Fos are activated by PKC isomers, probably due to activation of mitogen-activated protein kinase (MAPK) pathways including the JNK and ERK (reviewed in Ref. 45). Furthermore, PKC was suggested to indirectly regulate the phosphorylation state of c-Jun (46). As the JNK pathway and c-Jun autoregulation are not required for c-Jun induction by HU, it is conceivable that the ERK pathway may contribute significantly to c-Jun induction, possibly through c-Fos up-regulation, although its dimerization partner is yet unidentified. Furthermore, in correlation with the lack of a role for c-Jun in its own induction, phosphorylation of Ser-243, which has been suggested to be reduced upon elevation of c-Jun DNA binding capacity, was not reduced but actually increased by HU (data not shown).

In vivo experiments demonstrated that activities of the p53 and Rb pathways are important for drug-induced senescence (2). Genotoxic stress caused by exposure to chemotherapeutic drugs, including HU, up-regulates the activity of p53 and the CDK inhibitors p21^{cip1} and p16^{INK4a}, whose combined activity leads cells to enter senescence. Interestingly, AP-1 proteins c-Jun and JunB, which are up-regulated by HU, regulate these pathways in several ways. c-Jun was shown to affect the basal level of p53 and the induction of p21^{cip1} (22, 47). However, evidence suggests that HU-induced senescence can occur in the absence of p53 activity as K562 cells, which are deficient in p53, enter HU-induced senescence (43). Another study demonstrating enhanced senescence in thyroid anaplastic cancer cell lines by combined treatment with ionizing radiation and a pharmacologic JNK inhibitor, regardless of their p53 status (48), also suggests that in some cases, senescence can occur also in the absence of p53 induction. Nevertheless, in this study, we demonstrate that c-Jun and JunB proteins may possess antagonistic effects on the Rb pathway. p16^{INK4a}, a direct target of JunB, is induced by HU, and cyclin D1, a c-Jun regulated gene, is up-regulated in a c-Jun-dependent manner. No induction of cyclin D1 by HU is observed in the absence of c-Jun expression. Similarly, p16^{INK4a} is not induced when JunB expression is knocked down. The balance between these proteins may determine the cellular response to the chemotherapeutic drug, i.e. HU, arrests cellular proliferation and drives cells to senescence, whereas high levels of cyclin D1 may prevent it. Indeed, similar to the premature entrance of c-Jun-deficient MEFs to senescence (29, 30), c-Jun-deficient immortalized fibroblasts, in which cyclin D1 is not induced in response to HU exposure, or JunB-overexpressing cells are more sensitive to HU-induced senescence. Although inhibition of Rb via cyclin D1/CDK4-dependent phosphorylation seems to be a tempting explanation for the attenuation of HU-induced senescence, it is not necessarily an exclusive one. The ability of cyclin D1 to inhibit senescence induced by Rb overexpression is not totally dependent on its ability to enhance CDK4-dependent Rb phosphorylation (49). Furthermore, c-Jun itself interacts with Rb (50) but without known biological effects. An additional intriguing mechanism that may underlie the sensitivity of c-Jun-deficient cells to senescence is impairment in the ability to repair DNA damage (51), although the molecular mediators of the senescence in this case are yet to be identified.

Results from ectopic expression of cyclin D demonstrated that under restricting conditions, cyclin D1 overexpression may lead cells to apoptosis (52, 53). It is important to mention that cyclin D sensitizes cells to HU-induced apoptosis (54). These results correlate with the increased sensitivity to apoptosis of c-Jun-expressing cells. However, additional c-Jun target genes such as Fas may also contribute to c-Jun-dependent apoptosis (55). We and others demonstrated that c-Jun-deficient cells are also more resistant to UV-induced cell death (47, 56). Previous results, which demonstrate that senesced cells exposed to apoptotic stimuli such as UV radiation and hydrogen peroxide are more resistant to apoptosis, are in agreement with our results (57, 58). Overall, our data suggest that in addition to the activity of AP-1 proteins in proliferation and apoptosis, c-Jun and JunB may also have roles in the regulation of chemotherapy-induced senescence.

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