p53-independent Induction of p21\textsuperscript{WAF1/CIP1} Expression in Pericentral Hepatocytes following Carbon Tetrachloride Intoxication\textsuperscript{1}

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Abstract
The cyclin-dependent kinase, proliferating cell nuclear antigen, and stress-activated protein kinase/c-jun NH\textsubscript{2} terminal kinase inhibitor p21\textsuperscript{WAF1/CIP1} can induce G\textsubscript{1} arrest, and its expression coincides with the cessation of replication in many systems. We examined expression of p21 during the early stages of carbon tetrachloride intoxication in the mouse liver and observed a dramatic increase in p21 RNA levels between 4 and 8 h after administration. p21 expression, visualized by in situ hybridization, is induced in pericentral hepatocytes before carbon tetrachloride-induced necrosis. Examination of c-fos and c-myc expression patterns confirm that these immediate-early genes are induced in similar regions of the mouse liver. p21 induction is not dependent on p53; we observed similar levels and localization of p21 in wild-type and p53 null animals. Immunohistochemical localization of p21 and CCAAT/enhancer-binding protein expression shows that p21 protein accumulation is limited to a subset of CCAAT/enhancer-binding protein-positive hepatocytes. A second peak of perportal and intermediate zone-specific p21 gene expression, appearing 1–2 days after injection, is also p53 independent and may represent cell cycle checkpoints or postmitotic growth arrest. Sporadic p21 expression was also detected in pairs of hepatocytes distributed throughout the liver acini in healthy animals. Together, these data suggest several roles for p21 in the liver in response to toxicity, regeneration, and growth inhibition.

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Introduction
In the classical liver lobule, portal triads surround a central vein, defining a path of blood detoxification and bile flow within the organ. The portal triads contain a bile duct, portal vein, and portal artery. They serve as a dual source of blood, from the intestinal portal circulation via branches of the portal vein, and from the heart via the portal artery. The blood is detoxified and metabolically conditioned as it flows through sinusoids of hepatocytes to the central vein. The expression of many genes is governed by the location within the acinus, which is commonly divided into perportal, intermediate, and pericentral zones (zones I, II, and III, respectively; Reviewed in Ref. 1).

Although the adult liver does not normally turn over rapidly, it shares similarity with other epithelial tissues that undergo rapid renewal in that it is capable of regeneration (reviewed in Refs. 2 and 3). Compensatory growth is initiated after chemical or physical injury, ending when the liver has regained its original size. Hepatocytes have the ability to divide and appear to be primarily responsible for the generation of new hepatocytes in the adult liver during liver regeneration (4). Studies also suggest the existence of facultative stem cells near the portal triads, which can contribute to either bile duct or hepatocyte populations when the intrinsic capability of the differentiated hepatocytes to enter into cell division is blocked (5).

Carbon tetrachloride damages the liver as a result of its microsomal cytochrome P-450 catabolism, which generates Cl\textsuperscript{+} + CCl\textsubscript{3}{-}, free radicals (6, 7). These catalyze lipid peroxidation of the microsomal membrane, resulting in oxidative stress. The ensuing tissue damage occurs in a zone-specific manner within the liver. Approximately one-third of the liver parenchyma surrounding the central veins becomes necrotic, with degenerated nuclei, infiltration of polymorphonuclear lymphocytes, and hemorrhage (8). The damage is zone specific because pericentral hepatocytes express higher levels of several cytochrome P-450s than other hepatocytes (9, 10).

Liver regeneration following cell loss due to necrosis or partial hepatectomy is a tightly regulated process (2). The signals that regulate liver growth during regeneration must include factors that stop cell proliferation, once the appropriate size has been attained. One factor that is likely to regulate liver growth is the cyclin-dependent kinase inhibitor p21 (also known as WAF1, CIP1, SD1, and MDA-6; Refs. 11–15), which can induce G\textsubscript{1} arrest and block entry into S-phase by inactivating Cdk\textsuperscript{3} or by inhibiting activity of

\textsuperscript{3} The abbreviations used are: Cdk, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; C/EBP, CCAAT/enhancer-binding protein; RT-PCR, reverse transcription-PCR; DAPI, 4',6-diamidino-2-phenylindole; SAPK, stress-activated protein kinase; JNK, c-jun NH\textsubscript{2}-terminal kinases; TNF, tumor necrosis factor.
PCNA (reviewed in Ref. 16). It was demonstrated recently that targeted expression of a p21 transgene to the mouse liver can inhibit hepatocyte cell proliferation during normal development and during compensatory growth after partial hepatectomy (17).

To address the role of endogenous p21 during liver regeneration, we examined p21 expression following administration of carbon tetrachloride. Liver cell necrosis is a biologically relevant stimulator of liver regeneration and plays a role in a number of clinical conditions (18). We found that carbon tetrachloride-induced liver damage leads to two waves of p21 expression. The first occurs in the damaged pericentral region, in conjunction with early proto-oncogene expression. The second wave occurs later and is consistent with postmitotic expression. In the normal adult liver, we detected p21 expression in a few cells in a pattern that was also suggestive of rare postmitotic expression. Because the tumor suppressor protein p53 has been shown to activate p21 gene expression following DNA damage (11, 19–21), we examined p21 expression in p53-deficient mice following necrotizing injury. In these animals, the levels of p21 RNA are similar during both waves of expression, indicating that activation of p21 transcription is not p53 dependent. This is further confirmed by the similar patterns of 6-h peak p21 expression detected by in situ hybridization or immunohistochemistry in p53-deficient and wild-type animals, which rule out more localized p53 regulation. p21 expression in nuclei coincided with that of the transcription factor C/EBP-α, a possible activator of the p21 gene.

Results

**p21 Is Expressed in a Cell-specific Pattern in Normal Liver.** In many epithelial tissues, p21 expression is coincident with the cessation of cell replication. Its expression is induced as cells begin to undergo terminal differentiation, but it is absent from dividing cells and often decreases as terminally differentiated cells mature and migrate away from the proliferative zone (22). The liver is unique in that differentiated hepatocytes exit the cell cycle but are retained in the tissue and remain capable of replication. This temporary halt to replication provides an interesting model of cell cycle exit as opposed to terminal differentiation.

We examined p21 expression in the normal liver by in situ hybridization. Although p21 expression is undetectable in the majority of cells, it is expressed at relatively high levels in a few scattered hepatocytes. This result was obtained with both viral antigen-free and conventionally housed animals, with some variability in the number of positive cells detected. One conventionally housed animal yielded a particularly large number of scattered hybridizing cells (Fig. 1). The hybridizing nuclei are often paired and occur throughout all zones of the liver, matching the distribution expected if p21 is normally expressed in daughter cells after sporadic cell division to cause G1 arrest, but subsequently decreases. PCNA, which is directly inhibited by p21, has also been shown to be localized to the nuclei of sporadic hepatocytes during S phase preceding division (23, 24).

**Carbon Tetrachloride Intoxication Dramatically Increases the Number of p21-expressing Cells.** To determine if p21 plays a role in inhibiting the proliferative response of hepatocytes in response to toxin-induced damage, we examined p21 expression following administration of carbon tetrachloride by in situ hybridization. Because the peak of DNA synthesis following carbon tetrachloride damage has been reported to occur between 1 and 2 days (3, 25–27), we first examined p21 expression at 1, 2, 3, and 7 days after carbon tetrachloride injection (Fig. 2). Between 1 and 3 days, large numbers of p21-positive cells were visible in the periportal and intermediate regions of the liver lobule (zones 1 and 2), where hepatocytes have been shown to divide following carbon tetrachloride administration (25). p21 continues to be observed at high levels in pairs of cells, even 1 week after treatment (Fig. 2, G and H). Necrotic cells and cells of the immune system surrounding the central vein have very little if any signal for p21.

We next examined p21 expression at earlier time points, 1 to 12 h after carbon tetrachloride injection (Fig. 3). p21 gene expression was dramatically increased in an almost exclusively pericentral pattern, where evidence of cellular necrosis is visible 1 day after treatment. These cells represent a completely different cell population than that expressing p21 at 24 h and afterward. Pericentral expression appears to peak at 4–8 h after injection (Fig. 3, E–H). Levels of p21 mRNA expression were also examined by RT-PCR, and p21 RNA
levels were found to be greatest between 4 and 8 h after injection (Fig. 4). We also assayed the expression of S16 ribosomal protein, and the levels of RNA encoding this protein also changed during regeneration.

It has been shown that the early proto-oncogenes c-fos, c-jun, c-myc, c-Ha-ras, and c-Ki-ras are expressed in the pericentral and intermediate zones of the liver during the first 6 h following carbon tetrachloride injection in the rat (28). Because we suspect a relationship between these factors initiating the cell cycle and the regulatory effect of p21, and because the pattern in the rat did not appear so abruptly defined as our p21 hybridization, we examined the expression of c-fos and c-myc by in situ hybridization. Expression of c-fos appears in only some of the hepatocytes in the pericentral region at 1 h but can be seen in all pericentral hepatocytes by 2 h (Fig. 5). Expression returns almost to background by 4 h and is undetectable at 6, 8, and 12 h by in situ hybridization. Hybridization equal in intensity to that present near the central vein can also be seen near the periphery of the liver lobe in Fig. 5, A–D. It is interesting to compare Fig. 3F (p21 at 4 h) with Fig. 5B (c-fos at 1 h); although p21 expression is not as clearly focused, it appears to be induced in a fraction of cells throughout the pericentral region in a pattern that is similar to the early c-fos time point. The expression patterns of both factors expands to include all centrilobular hepatocytes at the next time point.

Expression of c-myc was examined at 1, 2, 4, 6, 8, and 12 h after carbon tetrachloride injection by in situ hybridization (Fig. 6). Other investigators have reported the induction of c-myc expression, with peak levels 2–3 h after partial hepatectomy and following carbon tetrachloride administration (28, 29). We observed two peaks of c-myc expression, the first at 2 h, and the second at 12 h after carbon tetrachloride injection (Fig. 6). Although we detected c-myc expression from 2 to 12 h in pericentral hepatocytes, the details of that pattern appear to change over time. At 6 and 8 h, the highest expression focuses near the edges of the expressing region, whereas at other time points, the pattern seems more homogeneous within the pericentral zone. c-myc expression was also surveyed by RT-PCR at these time points and after 1–7 days of regeneration, confirming high RNA levels during the first 12 h (data not shown). These similar, sharply restricted regions of p21 and c-myc expression may suggest that both are regulated by a common pathway.

To confirm the relevance of the increase in p21 RNA levels, we used an amplified immunohistochemical technique to visualize p21 protein (Fig. 7A). Whereas control levels of p21 were undetectable by this technique, at 6 h nuclear staining was clearly visible in a sharply delimited pattern among a subset of pericentral hepatocytes. Very little, if any, staining could be seen at 4 or 8 h (data not shown).

One candidate regulator for p21 is C/EBP-α, a transcription factor with leucine zipper dimerization motifs similar to c-myc, c-fos, and c-jun (30), which activates transcription via sequences conserved with c-fos and c-jun (31). C/EBP-α was shown to increase p21 levels 12–20-fold in HT1 cells, causing growth arrest that could be eliminated by expression of antisense p21 mRNA (32). C/EBP sites had been identified previously as important for rapid changes in gene expression
during carbon tetrachloride toxicity (33), and differential regulation of C/EBP factors is important during recovery after partial hepatectomy (reviewed in Ref. 34). We examined C/EBP-α expression to determine whether it might play a relevant role in regulating p21 expression following carbon tetrachloride damage. Livers at 6 h after carbon tetrachloride administration, stained with anti-p21 (Fig. 7B) and anti-C/EBP-α antibodies (Fig. 7C) and the DNA intercalating dye DAPI (Fig. 7D), show that a subset of pericentral hepatocytes contain both p21 and C/EBP in the nucleus. However, the pattern of C/EBP expression differs from p21 in that it is not restricted to the pericentral zone but extends with approximately equal frequency to all regions.

**Carbon Tetrachloride-induced p21 Expression Does Not Require the Presence of p53.** The p53 tumor suppressor gene encodes a transcription factor that activates expression of the p21 gene following DNA damage (11, 19–21). To determine if p53 is responsible for the dramatic induction
of p21 RNA expression following carbon tetrachloride treatment, p21 expression was examined by RNase protection and in situ hybridization in p53-deficient mice and genetically matched controls at 6 h after carbon tetrachloride injection. p21 is expressed in pericentral hepatocytes in both the p53-positive control animals (Fig. 8, A and B) and p53-null mice (Fig. 8, C and D). The lower level of p21 signal detected at 6 h in both the control and p53-deficient mice may reflect mouse strain differences, because a time course of p21 induction was performed only with the C3H/He strain and not the 129-SVJ strain that was the genetic background of the p53-null mice. The observation that p21 mRNA expression is induced in pericentral hepatocytes of p53-deficient mice indicates that p53 activity is not required for the early wave of p21 gene expression. Overall levels of p21 RNA are similar between p53-null and wild-type mice at several time points (Fig. 9). p21 RNA expression is clearly induced at 6 h, and increased expression is also detected at 24 and 48 h, although some variability is apparent between animals. p53 appears to play little if any role under these conditions.

Discussion
We have demonstrated two waves of p21 gene expression subsequent to carbon tetrachloride administration. The first induction occurs within 12 h of injury within cells most affected by the toxin; the second follows after days of regeneration in the surviving cells undergoing division. It is likely that each wave of p21 expression represents a barrier to cell division, because high levels of p21 are consistently associated with growth-inhibitory activities. p21 can block several Cdkks controlling progression through the cell cycle, interfere with PCNA-mediated polymerase delta starts (reviewed in Ref. 16), and inhibit the SAPKs, also known as JNKs (35). SAPK/JNK phosphorylation of c-Jun is important for the activation of many immediate-early gene promoters containing AP-1 (activator protein-1) sites, including c-fos.

Continuous overexpression of p21 as a transgene prevents hepatocyte division, even in response to partial hepatectomy, so that facultative stem cells or other nonexpression cells must suffice for hepatocyte production (17). However, p21 is also induced during the proliferative response to 70% partial hepatectomy in a biphasic pattern with broad peaks at 3–24 h and 48–72 h, separated by an interval of very little expression at 30–36 h (36). Because the response to partial hepatectomy involves highly synchronized cell cycles, this may be interpreted as a peak at G1, followed by low expression during S phase, and high expression afterward. In this case, p21 clearly does not block cell division altogether but may instead serve as a cellular checkpoint, timing mechanism, or assembly factor for cyclin-Cdk complexes during a regulated progression through cell division.

The second peak of p21 expression in surviving, actively dividing hepatocytes following carbon tetrachloride administration appears analogous to that seen with partial hepatectomy or in sporadic cells of the normal liver. In each case, p21 expression can be interpreted as an overall brake on the rate of regeneration, as liver mass returns to normal, or as a checkpoint in the cell cycle, where inappropriate division may be halted. Conceptually, these models are not distinct, but major questions remain concerning the phases of the cell cycle in which p21 may be expressed and which factors actually regulate the degree of expression or the final replication decision. Here we know only that pairs of hybridizing hepatocytes, presumably newly divided daughter cells, are present in normal liver, and that strong portal hybridization occurs as early as 24 h after tetrachloride treatment, when surviving hepatocytes are only beginning to react to pericen-
tral necrosis. This suggests that p21 is expressed during G₁, either before or after a hepatocyte division, but we cannot rule out expression or activity of p21 in other phases of the cell cycle.

The role for early, pericentral p21 expression is complicated by the necrotic death of the affected cells, which makes it unclear whether p21 serves as a growth inhibitor, as a normal cell checkpoint, or as an inhibitor of programmed cell death. We show that p21 expression is associated with an induction of immediate-early gene expression, which has been proposed to play an important role in priming hepatocytes for replication (37–39). Expression of c-fos is rapidly induced, followed by c-jun and c-myc. Interestingly, the hepatocytes undergoing compensatory hyperplasia in response to partial hepatectomy have been shown to induce proto-oncogene expression in much the same way that pericentral hepatocytes do in response to carbon tetrachloride (28, 29, 40–42). But in that case, c-jun expression as visualized by in situ hybridization is distributed throughout the liver lobule following partial hepatectomy (41).

It is possible for hepatocytes to divide without induction of c-fos, c-jun, or LRF-1, in the case of direct hyperplasia in response to the mitogen 1,4-bis[2-(3,5-dichloropyridyl)oxy]benzene (43). Therefore, p21 expression, even in such diverse systems as partial hepatectomy and carbon tetrachloride intoxication, does not necessarily indicate a regulatory role in hepatocyte division in general but could still represent a more specific response to some perception of damage by these cells. One candidate for such a signal of damage is TNF-α, which is induced within 6 h after carbon tetrachloride injection (44). Injection of neutralizing antibody to TNF-α abolishes SAPK/JNK activity and c-jun mRNA transcription measured 15 min after partial hepatectomy and decreases hepatocyte replication measured days after the procedure (45, 46). TNF-α treatment causes a 6-fold increase in p21 mRNA stability in KG-1 myeloid leukemic cells, an effect which does not require protein synthesis (47). Local elevation of TNF-α and c-fos has been observed within 30 min after impact trauma in rat spinal cord (48), and TNF-α is likely to act in a paracrine fashion in the liver (45). Thus, an early response of TNF-α to oxidative damage might determine the centrilobular expression pattern of p21.

Immediate-early gene expression appears to represent a conserved pathway of response to damage in cases where

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**Fig. 5.** c-fos expression is induced in pericentral hepatocytes that will undergo necrosis. Paired brightfield and darkfield views (4-day exposure) are shown for 1 h (A and B), 2 h (C and D), and 4 h (E and F). Time points at 6, 8, and 12 h reveal no detectable signal (data not shown). P, portal triad. In each of these photographs, the surface of the liver is at right. Bar, lower right, 50 μm.
Fig. 6. c-myc expression is induced in pericentral hepatocytes that will undergo necrosis. In situ hybridizations were performed with c-myc. Paired brightfield and darkfield views following 5 days of exposure are shown for 1 h (A and B), 2 h (C and D), 4 h (E and F), 6 h (G and H), 8 h (I and J), and 12 h (K and L) time points after carbon tetrachloride injection. P, portal triad; C, central vein. Bar, lower right, 50 μm.

regeneration is an option. Expression of p21 could be poised as a checkpoint in this process. By inhibiting SAPKs/JNKs, p21 could play a role in terminating the immediate-early response; for instance, we saw strong expression of c-fos at 2 h replaced by a similar strong expression of p21 at 4 h, with almost complete inhibition of c-fos expression. Simultaneously at 4 h, p34cdc2 (Cdk) and PCNA are already increased in level after carbon tetrachloride injection in rats (49), but high levels of p21, which specifically inhibits both of these proliferation markers, could hold S-phase entry in check. Through the combination of these activities, transient expression of p21 could enforce a pause in cell cycle progression, during which signals of cellular damage or replication halt, such as p53, TNF-α, or TGF-β, would have the opportunity to prolong p21 expression and halt the division permanently.

We found that p21 mRNA is expressed nearly homogeneously within the pericentral zone shortly following carbon tetrachloride administration. In contrast, p21 protein was detected in only a subset of the RNA-positive cells, and these cells also contained nuclear C/EBP-α. In a preadipocyte cell line, C/EBP-α causes a 12–20-fold increase in total
p21 levels but only a 3-fold increase in mRNA levels by greatly increasing the half-life of p21 protein in cycloheximide-treated cells (32). A similar influence here might account for the difference between p21 protein and RNA patterns. Mice in which C/EBP-α has been disrupted have livers with a morphology resembling regenerating liver following partial hepatectomy or pseudoglandular hepatocellular carcinoma (50). Interestingly, at 7 h after birth, C/EBP-α null mice express approximately 10-fold more c-jun, 6-fold more c-myc, and have 5–10 times more PCNA-positive cells, which appear to be in G1 or S phase. Conversely, overexpression of C/EBP-α in a hepatocyte cell line can block T-antigen-driven replication by one-third and largely abolishes c-myc induction by growth factors when the cell line is quiescent (51). In the context of a general antagonism between C/EBP-α and c-myc, p21 expression may signify an overall decision regarding cell proliferation.

Mice with a disruption of the p21 gene were generated, and they develop normally and do not develop tumors (52, 53). It will be of interest to determine whether mice deficient in p21 have an altered time course of immediate-early gene expression or allow some damaged liver cells to escape necrosis and replicate or undergo apoptosis, or undergo a greater degree of late DNA replication, following carbon tetrachloride administration.
Materials and Methods

Carbon Tetrachloride Injection and Tissue Processing. Viral antigen-free adult male p53-/- and C3H/He mice were obtained from The Jackson Laboratory (Bar Harbor, Me). Conventionally housed ICR mice were from a breeding colony established at the University of Illinois at Chicago. Mice were injected i.p. with 10 μg body weight of a 10% solution of carbon tetrachloride in mineral oil, or mineral oil alone, and sacrificed at the times indicated. Portions of each liver were embedded in OCT cryopreservation medium for sectioning or were frozen for RNA preparation. Total RNA preparation involved tissue homogenization in guanidine thiocyanate solution with 2-mercaptoethanol, followed by CsCl gradient centrifugation (54).

In Situ Hybridizations. In situ hybridizations were performed as described (55). A 728-bp p21 cDNA fragment, flanked by BamHI and HindIII sites in pBluescript II SK, was a gift from Wafik El-Deiry (University of Pennsylvania, Philadelphia, PA). CDNA clones encoding mouse c-fos and c-myc (56) were a gift from L. F. Lau (University of Illinois at Chicago); an NH2-terminal encoding Cat/BamHI fragment of c-myc and a COOH-terminal encoding SatI/EcoRI fragment of c-fos, each approximately 900 bp in length, were subcloned into pBluescript II SK. Each construct was used for T3 and T7 in vitro run-off transcription, terminated at an appropriate restriction enzyme site. All templates were purified on 5% acrylamide gels to remove nonlinearized molecules. After in vitro transcription, the probes were hydrolyzed to 150-bp fragments in sodium carbonate buffer at 60°C for better tissue penetration. After hybridization and washing, slides were dipped into undiluted NTB-2 emulsion, exposed for 5-9 days, and then stained with H&E through the emulsion.

Quantitative RT-PCR. For synthesis of cDNA, we used the SuperScript Preamplification system (Life Technologies, Inc.). Two μg of total cellular RNA were annealed with 50 ng of random hexanucleotide primer at 70°C for 10 min. CDNA was synthesized in a 20-μl volume containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 10 mM DTT, 500 μM deoxynucleotide triphosphates, and 200 units of SuperScript II reverse transcriptase at 42°C for 50 min, after which we added 2 units of RNase H. CDNA product (2.5 μl) was added to 30 μl of PCR reaction mixture that contained 200 μM deoxynucleotide triphosphates, 2.5 mM MgCl2, 75 pmol of primers, and 1.5 units of AmpliTaq polymerase (Life Technologies, Inc.). Before the reaction, AmpliTaq polymerase was preincubated for 10 min with TaqStart antibody (Clontech). Each cycle of PCR included 45 s of denaturation at 94°C for 1 min of primer, annealing at a different temperature for each primer, and 1 min of extension/synthesis at 72°C. For each combination of primers, the kinetics of PCR amplification were studied, the number of cycles corresponding to plateau were determined, and PCR was performed within the exponential range. For amplification of mouse RNA, we used the following primers for p21 (upstream primer, AGGCT-GAAAGCTGTGATG; downstream primer, AAATTTCCAGGTTCTCGG; annealing temperature, 65°C; 30 cycles; product, 228 bp) and mouse S16 ribosomal protein, as internal control (Ref. 57; upstream primer, AGGAGCGAGTTGCTGTGG; downstream primer, GCTAC-CAGGGCTTTGAGATG; annealing temperature, 72°C; 28 cycles; product, 102 bp). Keratin 8 and β2-microglobulin were also used as internal controls in some experiments (data not shown). PCR products were separated on a 2% agarose gel and stained with ethidium bromide. Gels were photographed in UV light using a Hitachi KPM1-U camera, and bands corresponding to each specific PCR product were quantitated using NIH image (available by ftp from zippy.nimh.nih.gov).

Immunochemistry. Immunological staining of p21 and C/EBP-α was enhanced using TSA-Indirect (DuPont/NE). Briefly, frozen sections were fixed in 4% parafomaldehyde in PBS for 2 min, washed in PBS, blocked with 2% goat serum in wash buffer (0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl, and 0.05% Tween 20) for 40 min. Sections were then incubated overnight at 4°C with anti-WAF1 (Ab-5) polyclonal rabbit antibody (OncoGene Research) at 1:200 dilution or anti-C/EBP-α (14A4) polyclonal rabbit antibody (Santa Cruz Biochemicals) at 1:200 to 1:500 dilution in wash buffer with 2% goat serum. After several washes and a 1-h incubation with biotinylated goat anti-rabbit IgG secondary antibody (1:250; Vector Laboratories), the steps for incubation with streptavidin-horseradish peroxidase and biotinyl tyramide reaction were done in accordance with the manufacturer's instructions. Deposited biotinyl tyramide was visualized with fluorescein or rhodamine avidin (DGS grade, 1:500 dilution; Vector), washed, and mounted with Vectashield medium.

For double staining, the procedure was performed as above for fluorescein visualization of the first antibody; then avidin and biotin blocking solutions (Vector) were used to suppress further avidin/biotin interactions from the first staining procedure, and the procedure was repeated to rhodamine visualization and mounting. To counter false results from cross-hybridization, both possible orders of hybridization were examined and compared to single-antibody stains.

RNase Protection Assays. p21 mRNA expression was analyzed by RNase protection assay using an [32P]UTP-CTP labeled antisense riboprobe transcribed from the pBluescript SK II + plasmid (Stratagene) using T7 polymerase (Promega Corp.) terminated at the SphI site present in the 3' noncoding sequence of the p21 CDNA clone described for in situ hybridizations and including antisense sequence from bases 617 to 733 of p21 cDNA (NMU24173; Ref. 58).

Twenty μg of each total RNA sample or an equal amount of Baker's yeast tRNA (Boehringer Mannheim, Indianapolis, IN) was precipitated with ethanol and resuspended in 30 μl of hybridization buffer containing 2 × 106 cpm of probe. Samples were hybridized 12-18 h at 55°C and then treated for 30 min at 37°C with 350 μl of RNase digestion buffer (10 μg Tris-Cl (pH 7.5), 5 mM EDTA, and 300 mM NaCl) containing 0.23 unit RNase A and 25 units RNase T1 per sample (Boehringer Mannheim). Proteinase K digestion, phenol:chloroform extraction, ethanol precipitation, and analysis on denaturing acrylamide gels were done as described (59).

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