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Overexpression of Cyclin D1 Inhibits TNF-Induced Growth Arrest

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Abstract Although activated macrophages destroy cancer cells more effectively than normal cells, the facility to escape activated macrophages is a characteristic of tumor cells. One of the mechanisms responsible for the specific killing of tumor cells by macrophages is the production of the cytokine tumor necrosis factor alpha (TNF). Therefore, resistance to TNF may provide such cancer cells a selective advantage against host elimination. In the present work we explore the possibility that cyclin D1 overrides the cytostatic effect of TNF. We show that TNF induces $p21^{waf1}$ protein in malignant melanoma A375 cells and its binding to CDK2/4 and 6 proteins, and thereby inhibiting the activity of these complexes. This inhibition leads the cells to a G1 arrest. Overexpression of cyclin D1 in these cells makes them insensitive to TNF treatment with the recovery of CDK activity, however, is unable to overcome the inhibitory action of etoposide blocking the cells on G2/M. The bypass of TNF-induced G1 arrest seems to be related to the increase in the stability of cyclin D bound CDK complexes, increasing the total amount of CDK2/4 and 6 complexes and leading to a functional down titration of the $p21^{waf1}$ molecules. In these conditions the TNF-induced increase of $p21^{waf1}$ is not sufficient to inhibit the high amount of cyclin D-bound complexes. This hypothesis is supported by the fact that a reduction in the levels of $p21^{waf1}$ protein, induced by the expression of a mRNA antisense against $p21^{waf1}$, is also able to bypass of TNF-induced arrest. Our results confirm that $p21^{waf1}$ has an essential role in TNF-induced arrest and that the deregulation of cyclin D1 may be one of the mechanisms to escape physiological signals to restrict tumoral growth. J. Cell. Biochem. 89: 484–499, 2003.

Key words: TNF; cell cycle; cyclin D; growth arrest; cancer

Tumor necrosis factor (TNF) is one of the most important cytokines produced by almost all cell types of the immune system [Beutler and Cerami, 1989; Vilcek and Lee, 1991]. TNF mediates a wide variety of biological effects that include activation of neutrophils and monocytes [Shalaby et al., 1985; Feinman et al., 1987], mediation of inflammatory response [Beutler and Cerami, 1988], endotoxic shock [Tracey et al., 1988], and cytostatic and/or cytotoxic effects in tumor cells in vitro and in vivo [Carswell et al., 1975; Sugarman et al., 1985; Haranaka et al., 1986; Wanebo, 1989; Balkwill et al., 1990; Hieber and Heim, 1994]. This range of biological activities depend on the cell type, stage of differentiation, and transformation state. It has been estimated that 40% of tumor cell lines are growth inhibited by TNF in vitro [Goeddel et al., 1986], but not normal cells from lung, colon, or fetal skin [Sugarman et al., 1985]. The pleiotropic effects of TNF are mediated by two distinct but structurally related cell surface receptors [Baker and Reddy, 1996]. These receptors are members of a superfamily, which includes NGFr, CD27, CD40, OX40, and Fas. TNFr are expressed in most if not all cell types, and the anticellular effects of TNF are normally paracrine.

A variety of cellular targets and metabolic or molecular changes have been linked to the cytotoxic effect of TNF, which includes DNA fragmentation, changes in lipid metabolism, lipid peroxidation, and increases in prostaglandin E production. TNF can also induce the phosphorylation of EGF receptor and molecules

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such as hsp 27 and hsp 70, NFkB and the eukaryotic initiation factor eIF 4E, activation of phospholipases A2 and D, sphingomielinase and ICE protease. Overexpression of Hsp 27 and Hsp 70 proteins as well as Bcl2, E1B 19k, cathepsin D, MnSOD or B/c-sis genes protect cells against TNF induced cytotoxicity [for review see Baker and Reddy, 1996; Schulze-Osthoff et al., 1998; Aggarwal, 2000; Chen and Goeddel, 2002; MacEwan, 2002]. Recently it has been shown that the activation of the PI3K-AKT pathway also induces loss of sensitivity to TNF apoptotic effect [Ozes et al., 1999, Burow et al., 2000]. However, other oncogenes as ras, c-myc and E1A have been shown to induce cellular susceptibility to the cytotoxic action of TNF [Seliger et al., 1988; Fernandez et al., 1994; Chang et al., 1999].

However, although TNF shows well-documented apoptotic activity on tumor cells, it also produces cytostatic effects on other cell lines with concomitant G1 arrest [Jeoung et al., 1995; Harvat and Jetten, 1996; Shiohara et al., 1997; Merli et al., 1999]. At G1 checkpoint (Restriction point) both positive and negative external signals are integrated into the cell cycle and, depending on the balance of external signals, the cell commits itself to another round of DNA replication [Hunter and Pines, 1994; O'Connor, 1997]. In mammalian cells, D-type cyclins and their partners, primarily CDK4 and CDK6 [Draetta, 1994] regulate the G1 checkpoint. Cyclin D can also bind CDK2 although the complex formed lacks CDK activity [Higashi et al., 1996]. The D type cyclins are inducible by growth factors [Won et al., 1992]. This has lead to the hypothesis that D-type cyclin synthesis is a sensor for proliferation signals [Sherr, 1995]. And, therefore, deregulation of D-cyclins render cell cycle progression less dependent on growth factors and contribute to oncogenesis [Xiong et al., 1992b, 1993; Jiang et al., 1993; Imoto et al., 1997].

Overexpression of cyclin D1 confers transformed properties on stabilized fibroblasts [Jiang et al., 1993] and cooperates with oncogenes to transform primary cells [Hinds et al., 1994]. Moreover, overexpression of cyclin D1 alone under the control of the MMT virus LTR in transgenic mice results in mammary hyperplasia and carcinoma in females [Wang et al., 1994]. Cyclin D1 overexpression is a frequent event in a variety of human cancers. In humans, cyclin D1 is encoded by the *CCND1* gene on chromosome 11q13 [Xiong et al., 1992a] which has been identified as the *PRAD1* oncogene [Withers et al., 1991]. In centrolytic B cell lymphomas cyclin D1 is overexpressed as a result of a translocation at the *BCL1* breakpoint locates the cyclin D1/*PRAD1* gene under the inmmunoglobulin heavy chain enhancer [Withers et al., 1991]. In mantle cell lymphoma cyclin D1 is reported to be overexpressed in 100% of tumors [Campo et al., 1999] while in multiple myeloma overexpression occurs in one third of the tumors [Troussard et al., 2000; Rasmussen et al., 2001]. *Cyclin D1/PRAD1* is dramatically overexpressed in certain parathyroid adenomas where the chromosomal inversion inv(11)(p15;q13) locates the cyclin D1 gene under the control of the parathyroid hormone gene promoter. Cyclin D1 is also overexpressed in a proportion of breast, gastric, and oesophageal carcinoma as a result of amplification of the 11q13 region [Donnellan and Chetty, 1998].

Transition through G1 checkpoint is also regulated by the integration of cytostatic signals, such as TGFβ, ILs, IFNs. These signals induce CDK inhibitors, that in some cases displace the D cyclin from its CDK binding (INK4 family of CDK inhibitors, composed by p16INK4a, p15INK4b, p18INK4c, and p19INK4d) or inhibit the CDK/cyclin complex by binding more than one molecule of CKI to the complex (CIP/KIP family, composed by $p21^{waf1}$/cip1, p27Kip1, and p57Kip2) [for review see Carnero and Hannon, 1998; Sherr and Roberts, 1999].

It has been shown that many CDK inhibitors are induced in response to growth arrest stimulus. IFNγ-induced growth arrest has been related to a p53-independent activation of $p21^{waf1}$ [Harvat and Jetten, 1996]. TGFβ treatment of growth factor stimulated cells override the mitogenic stimulation by preventing the decline in $p27^{kip1}$ levels [Polyak et al., 1994]. TGFβ also induces $p15^{ink4b}$ and $p21^{waf1}$ all of them concomitant with the induction of G1 arrest [Reynisdottir et al., 1995]. Jeoung et al. [1995] showed that TNFα induces G1 arrest in MCF7 cells which has been related to $p21^{waf1}$ induction in a p53 dependent way, and this induction correlated with CDK inhibition. $p21^{waf1}$ induction was also observed by other groups in ME180 cervical cancer cells and MG-63 osteosarcoma cell line [Shiohara et al., 1997; Merli et al., 1999].

In the present work we explore the possibility that cyclin D1 overrides the cytostatic effect of

TNF and contributes to escape from physiological signals to restrict tumoral growth. We show that TNF induces $p21^{waf1}$ protein in malignant melanoma A375 cells, increasing the binding of $p21^{waf1}$ to CDK complexes, but not to PCNA. This binding correlates with the inhibition of the activity of the complexes and concomitant G1 arrest. The overexpression of cyclin D1 in A375 cells makes them insensitive to TNF treatment with the recovery of CDK activity. The bypass of TNF-induced G1 arrest seems to be related to and increase in the stability of cyclin D bound CDK complexes, and therefore a down titration of the $p21^{waf1}$ molecules bound to these complexes. This hypothesis is supported by the fact that antisense fragments against $p21^{waf1}$ bypass the TNF-induced arrest.

MATERIALS AND METHODS

Cell Culture

A375 malignant melanoma cells from ATCC were maintained in DMEM (Gibco) containing 10% fetal bovine serum (Sigma). Cultures were selected when indicated with 75 μg/ml hygromycin (Calbiochem), 400 μg/ml G418 (Sigma) or 2 μg/ml Puromycin (Fluka). To perform the gene transfer using ecotropic competent retroviruses, A375 cells were transfected with the murine receptor and a mass culture expressing the receptor used for the following experiments. When indicated, cells were treated with purified human recombinant TNFα (Gibco-BRL).

Retroviral-Mediated Gene Transfer

Packaging Phoenix cells were plated in a 10 cm dish, incubated for 24 h and then transfected by calcium phosphate precipitation with 20 μg of the retroviral plasmid (16 h at 37°C). After 48 h, the virus-containing medium was filtered (0.45 μm filter, Millipore) and supplemented with 8 μg/ml polybrene (Sigma) and an equal volume of fresh media. Target fibroblasts were plated at 8×10^5 cells/10 cm dish and incubated overnight. For infections, the culture medium was replaced by the appropriate viral supernatant, the culture plates were centrifuged (1 h, 1,500 rpm) and incubated at 37°C for 16 h. The infected cell population was purified using the appropriate selection.

Immunoblot Analysis

Cells were washed twice with ice-cold PBS and lysed in NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl pH 8.0, 1 mM PMSF, 1 μg/ml Leupeptin, 25 μg/ml Aprotinin, 1 mM EDTA). After 15 min on ice, samples were vortexed (5 min at 4°C) and cleared by centrifugation. The proteins were detected in total extracts using anti-human rabbit polyclonal antibodies generated at David Beach Laboratory [see Xiong et al., 1992b, 1993; Zhang et al., 1994], except the following proteins, PCNA (PC10 from Santa Cruz), cyclin A (C-19 from Santa Cruz) and pRb (SC-50 from Santa Cruz). In all cases specific proteins were detected using biotinylated secondary antibodies (Amersham) followed by HRP-conjugated streptavidin and ECL.

Immunoprecipitations were performed with 500 μg of total protein (Bio-RAD protein assay) followed by binding to protein A using anti-CDKs, anti-cyclins or anti-$p21^{waf1}$ antibodies (generated at David Beach Laboratory) which have been used for immunoblotting before. After several washes with lysis buffer the immunoprecipitated proteins were separated on SDS–PAGE, transferred to nitrocellulose membranes (Millipore). Bound proteins were detected by using the indicated specific antibody, and developed using biotinylated secondary antibodies (Amersham) followed by HRP-conjugated streptavidin and ECL.

CDK2 Kinase Assay

Immunoprecipitations were performed with 500 μg of total protein (BIO-RAD protein assay) using the anti-CDK2 antibodies (generated at David Beach Laboratory) followed by binding to protein A. Immunoprecipitates were washed several times with lysis buffer, twice with kinase assay buffer (20 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DDT) and then assayed for its ability to phosphorylate H1 histone as it has been described by Zhang et al. [1994]. Briefly, protein A-beads suspensions were brought to 20 μl with a solution containing 2.5 μg of Histone H1 (Boheringer) and 10 μCi of γ-32P-ATP (3000 Ci/mmol). Reaction mixtures were incubated for 15 min at room temperature and resolved on 10% SDS–PAGE gels. H1 kinase activity was revealed by autoradiographic exposure.

FACS

Cells were seeded at low density (aprox. 5%) in 10 cm dishes, grown until 70–80% confluence was reached and then treated with the indicated

agent for 24 h. Cells were trypsinized, fixed with 70% methanol, and washed twice with PBS containing 0.1% FCS. After incubation in PBS supplemented with RNAse (10 μg/ml) and propidium iodine (5 μg/ml) for 30 min the DNA content was analyzed by FAC Scan.

Growth Assays

Twenty-four hours after 10^4 cells were seeded in 2.5 cm dishes in triplicate samples (Day 0) medium was removed and replaced with culture medium in the presence of TNF at indicated concentrations. Controls remained untreated. When indicated, cells were fixed with 0.5% glutaraldehyde and stained with 1% crystal violet. After extensive washing, crystal violet was resolubilized in 10% acetic acid and quantified at 595 nm as a relative measure of cell number. Absorbance at Day 0 represent the number of imput cells.

$p21^{waf1}$ Antisense

p21 mRNA antisense fragment was generated by PCR from a human cDNA template. The resulting p21 fragment was cloned in antisense orientation in MARX retroviral vector [Carnero et al., 2000].

RESULTS

Cytostatic Effect of Tumor Necrosis Factor

TNF induces growth arrest or cytotoxicity on a large number of transformed cell lines. In A375, a human melanoma cell line, in absence of protein synthesis inhibitors, TNF induces cytostatic effects in a dose-dependent way in the range of 0.1–200 ng/ml (Fig. 1A). At 100 ng/ml, TNF completely blocks the growth of A357 cells (Fig. 1B). In this cell line, the growth arrest induced by TNF treatment occurs in G0–G1 phase of the cell cycle (Fig. 1C). Cell viability was not impaired in any of the experiments (Fig. 1C, and data not shown). The observed cytostatic effect is in accordance with the growth arrest induced by TNF in vitro on other tumor cell lines [Pusztai et al., 1993; Jeoung et al., 1995; Harvat and Jetten, 1996; Shiohara et al., 1997].

TNF Induces $p21^{waf1}$ Association to CDK Complexes

Since the G1 to S phase transition is driven by CDK complexes, we analyzed the impact of TNF treatment on these cell cycle regulators in A375 cells. Exponentially growing cells were treated with 100 ng/ml of human recombinant TNF during 24 h. Figure 2A shows that TNF induces an increase of the CDK inhibitor $p21^{waf1}$ in contrast to other cell cycle regulatory proteins analyzed (Fig. 2A). These results agree with previously published results of TNF effects in the osteosarcoma cell line MG-63 [Merli et al., 1999]. We did not detect p16INK4a protein confirming a previous work which reported a mutation within the p16INK4a gene in A375 cells that creates a stop codon [Stott et al., 1998]. Furthermore, p15INK4b was not detectable (not shown), which may indicate a deletion of the locus as it has been reported for p14ARF [Stott et al., 1998].

To study the cellular context of $p21^{waf1}$ we performed immunoprecipitations of the CDK complexes and analyzed the association of related proteins to these complexes. A375 cells were grown to 70–80% confluence and treated for 24 h with 100 ng/ml of TNF. Equal amounts of total cell lysate were immunoprecipitated with antibodies specific for CDK2, CDK4, and CDK6 proteins, respectively. Complex associated regulators were examined by Western blotting of the immunoprecipitates using specific antibodies against the bound proteins. $p21^{waf1}$ proteins associated with CDK complexes increased when cells were treated with TNF (Fig. 2B,C). Conversely, $p27^{kip1}$, a member of the p21 family of CDK inhibitors was barely detectable in the CDK immunoprecipitates from A375 cells (data not shown) suggesting that its association with the complexes was minimal. In the TNF-sensitive breast carcinoma cell line MCF7 $p21^{waf1}$ also increases its association with CDK complexes after TNF treatment, while $p27^{kip1}$ was clearly detectable and mainly associated with CDK4 without variations after TNF treatment (data not shown).

Additionally, we analyzed the association of the positive regulators cyclin D1 and cyclin A to CDK2/4 and 6 complexes and cyclin E to CDK2 after TNF treatment. In A375 cells, cyclin D1 seems to be mainly bound to CDK4 and 6, but binding is also observed to CDK2 (Fig. 2B). Cyclin D1 association to CDK2/4/6 slightly increases after TNF treatment (Fig. 2B). Conversely, cyclins A and E are only associated with CDK2 and no significant variations were observed upon TNF treatment (Fig. 2B).

$p21^{waf1}$ is a dual specificity inhibitor which aside from binding to CDKs associates with the

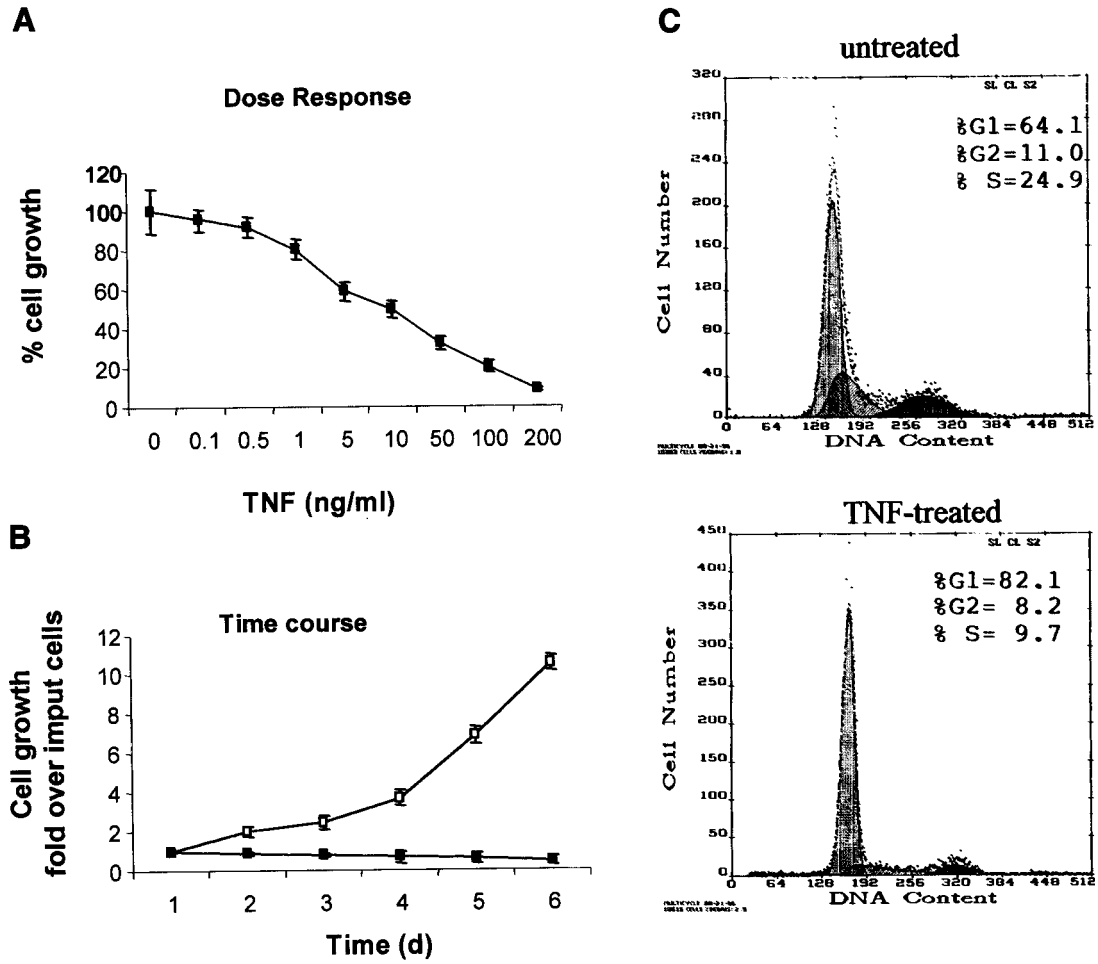

Fig. 1. Cytostatic effect of TNF in A375 melanoma cells. **A**: Dose response effect of TNF treatment. Cells (10^4) were seeded in 2.5 cm dishes in triplicate samples. After 24 h, medium was changed (Day 0) and the indicated dose of TNF added. After 3 days cells were fixed and stained with crystal violet. After extensive washing, crystal violet was resolubilized in 10% acetic acid and quantified at 595 nm as a relative measure of cell number. Values are expressed as the percentage of cell growth. Untreated control cells represent 100% of cell growth. Zero percent refers to the number of cells at Day 0. The data are presented as mean from triplicate samples; bars, ± SD. **B**: Time course of A375 cells grown in the presence of TNF. Twenty-four hours after seeding of 10^4 cells in 2.5 cm dishes the growth medium was changed (Day 1) and 100 ng/ml TNF was added to the samples. The growth of TNF-treated (■) or untreated (□) samples was stopped at the indicated time points and analyzed by staining with crystal violet. After extensive washing, crystal violet was resolubilized in 10% acetic acid and quantified at 595 nm as a relative measure of cell number. Values are expressed as the average of growth normalized to the number of input cells (Day 1). The data are presented as mean from triplicate samples; bars, ± SD. **C**: TNF induces G1 arrest. Cells were seeded at low density (approx. 5%) in 10 cm dishes and grown until 70–80% confluence was reached. Cells were then treated with 100 ng/ml TNF for 24 h and subjected to PI staining. Cell cycle phases were detected analyzing the DNA contents of untreated control cells (untreated) and cells treated with TNF (TNF-treated) using FACS.

DNA replication factor PCNAs blocking its ability to induce DNA synthesis [Waga et al., 1994]. However, immunoprecipitation using specific antibody against $p21^{waf1}$ revealed no significant increase of PCNA associated with $p21^{waf1}$ in A375 cells upon TNF treatment (Fig. 2D).

In order to examine the effect of TNF treatment on the activity of the CDK2, CDK4, and CDK6 complexes we performed analysis of H1 histone phosphorylation and monitored the pRB phosphorylation state. We immunoprecipitated CDK2 complex from cells treated with 100 ng/ml TNF for 24 h and assayed their ability to phosphorylate H1 histone. TNF treatment inhibited H1 kinase activity associated with CDK2 dramatically (Fig. 2E). Since pRB is the main substrate of CDK4 and CDK6, the endogenous phosphorylation state of pRB upon TNF treatment may reflect cytokine induced changes

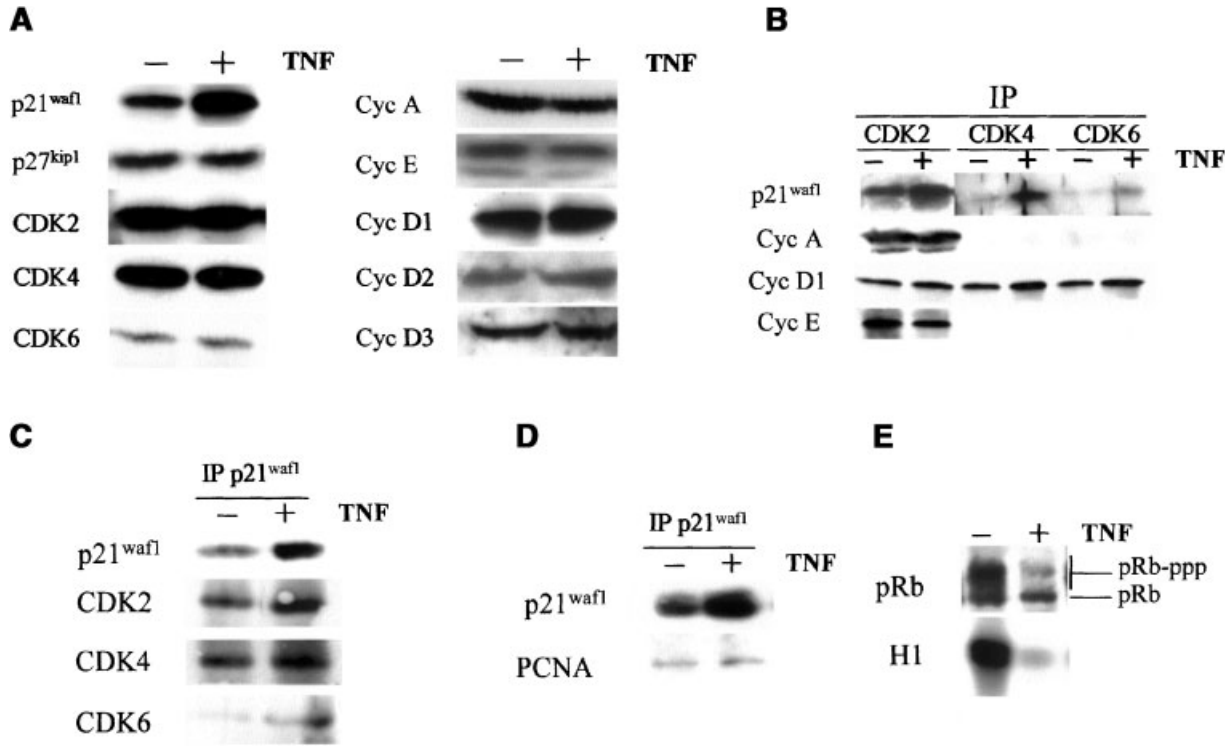

Fig. 2. Effect of TNF treatment on cell cycle related proteins. **A**: Levels of cell cycle related proteins after TNF treatment. Cells were seeded at low density (approx. 5%) in 10 cm dishes, grown until 70–80% confluence was reached and then treated (+) or not (–) with 100 ng/ml TNF for 24 h. Cells were then lysed and 100 μg of cellular proteins resolved in 12% SDS–PAGE. Each protein was detected by immunoblotting using the indicated antibodies. **B**: Variation in $p21^{waf1}$ bound to CDK complexes after TNF treatment. Cells were seeded and processed as described before (A). CDKs were immunoprecipitated from equal amount of lysate in all samples (500 μg of total protein) using specific antibodies (IP; CDK2, CDK4, CDK6). Proteins bound to the immunoprecipitated complexes were separated by SDS–PAGE. Each protein was detected by immunoblotting using the indicated antibodies. **C**: Variation in CDKs bound to $p21^{waf1}$ after TNF treatment. Cells were seeded and processed as described before (A). $p21^{waf1}$ proteins were immunoprecipitated from equal amount of lysate in all samples (500 μg of total protein) using a specific antibody. CDK proteins bound to the immunoprecipitated complexes were separated by SDS–PAGE. Each protein was detected by immunoblotting using the indicated antibodies. **D**: TNF treatment does not increase the binding of PCNA to $p21^{waf1}$. Cells were seeded and processed as described before (A). $p21^{waf1}$ was immunoprecipitated from equal amount of lysate in all samples (500 μg of total protein) using a specific antibody (IP; $p21^{waf1}$). Proteins bound to the immunoprecipitated $p21^{waf1}$ were separated by SDS–PAGE. $p21^{waf1}$ and PCNA were detected by immunoblotting using the indicated antibodies. **E**: TNF treatment inhibits CDK2 activity and pRb phosphorylation. Cells were seeded and processed as described before (A). pRb phosphorylation was detected by the mobility shift observed in the hyperphosphorylated protein (pRb-ppp) on Western blot. One hundred micrograms of total protein lysates were resolved in 6% SDS–PAGE and transferred to a nitrocellulose membrane. pRb protein was identified using a specific antibody. CDK2 activity was identified by its H1 kinase activity. CDK2 was immunoprecipitated from equal amount of lysate in all samples (500 μg of total protein) using a specific antibody. The immunoprecipitates were assayed for its capability to phosphorylate histone H1 (with 32P-ATP) and the band for H1 protein resolved in SDS–PAGE.

in the activity of these complexes. In order to study the activity of CDK4 and CDK6, we analyzed mobility shift of hyperphosphorylated pRB protein from lysates of exponentially growing A375 cells. As Figure 2E shows, a big portion of pRB proteins is hyperphosphorylated in untreated A375 cells whereas the level of phosphorylation is significantly decreased after treatment with 100 ng/ml of TNF suggesting that the cytokine reduces the activity of the CDK complexes 4 and 6.

Ectopic Expression of Cyclin D1 Renders A375 Cells Insensitive to TNF

Since major regulatory events leading cells to G1 arrest occur at CDK level by inhibitory proteins, the deregulated expression of G1 phase cyclin might cause the loss of cell cycle control, as it has been reported previously for TGFβ [Okamoto et al., 1994]. In the case of TNF, deregulation of the cell cycle may contribute to the protection from its cytostatic effect. Cyclin

D1 has been shown to be associated with several proteins including cdc2, CDK2, CDK4, CDK5 and CDK6, but there is a large body of evidence that cyclin D1 promotes its essential role in controlling the G1 phase of the cell cycle by activating CDK4 and CDK6 [Draetta, 1994; Sherr, 1995]. In order to examine the implication of the cyclin D1 in the resistance to TNF-induced cell cycle arrest we overexpressed human cyclin D1 cDNA under retrovirus mediated transduction in A375 cells. After selection, the culture was analyzed for cyclin D1 protein expression. Total lysates of cells infected with plasmid alone (A375) or carrying the human cyclin D1 cDNA (A375-D1) were analyzed (Fig. 3A). High levels of cyclin D1 were expressed in A375-D1, but not in the control cells. To test if the cells overexpressing cyclin D1 still respond to TNF, we analyzed the $p21^{waf1}$ induction in response to the cytokine. Figure 3A shows that ectopic expression of cyclin does not alter the increase of $p21^{waf1}$ protein upon TNF treatment, indicating that cyclin D1 overexpressing cells still respond to TNF.

Conversely, the overexpression of cyclin D1 had dramatic effects on the biochemical activity of the CDK complexes. Cyclin D1-overexpression abrogated the TNF induced decrease of pRB phosphorylation (Fig. 3B). Furthermore, the dramatic reduction of H1 kinase activity of CDK2 immunoprecipitates upon TNF treatment was completely blocked in cyclin D1-overexpressing cells (Fig. 3C).

In addition, we analyzed the effect of the overexpression of cyclin D1 on the physiological sensitivity to TNF treatment. FACS analysis revealed that overexpression of cyclin D1 suppresses cell cycle arrest at G1 after TNF treatment (Fig. 4A). Treatment of exponentially growing A375 cells with 100 ng/ml of TNF for 24 h increased the G0-G1 population to 80% and decreasing the cells in S phase to 10%. Cells overexpressing cyclin D1 display a small increase in G0-G1 cells, but after 24 h of TNF treatment the G0-G1 population of cells is comparable to exponentially growing control cells. As shown in Figure 4A we could not detect sub G1 population in A375 or in A375-D1 cells in the presence or absence of TNF. This result indicates that cycling cells overexpressing cyclin D1 did not undergo apoptosis upon TNF treatment.

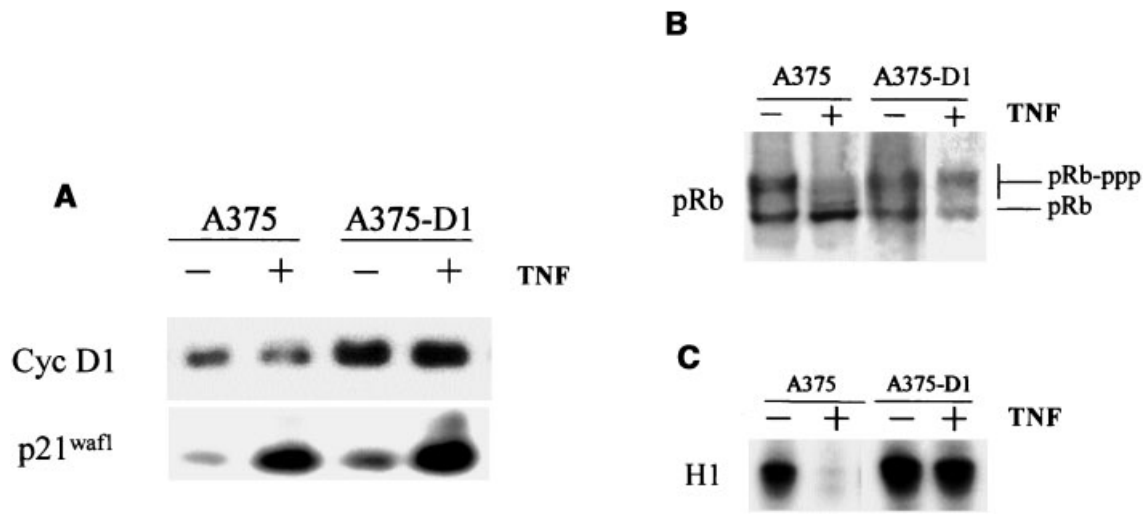

Fig. 3. Overexpression of cyclin D1 maintains CDK activity in the presence of TNF. **A**: Overexpression of cyclin D1 does not inhibit TNF-induced increase of p21. A375 parental cells (A375) and A375 cells ectopically overexpressing cyclin D1 (A375-D1) were seeded at low density (approx. 5%) in 10 cm dishes, grown until 70–80% confluence was reached and then treated (+) or not (−) with 100 ng/ml TNF for 24 h. Cells were then lysed and 100 μg of cellular proteins resolved in SDS–PAGE. Cyclin D1 and $p21^{waf1}$ were detected by immunoblotting using the indicated antibodies. **B**: TNF treatment does not inhibit pRb phosphorylation in the presence of high levels of cyclin D1. Cells were seeded and processed as described before (A). pRb phosphorylation was detected by the mobility shift observed in the hyperphosphorylated protein (pRb-ppp) on Western blot. One hundred micrograms of total protein lysate were resolved in 6% SDS–PAGE and transferred to a nitrocellulose membrane. pRb protein was identified using a specific antibody. **C**: TNF treatment does not inhibit CDK2 activity in the presence of high levels of cyclin D1. CDK2 activity was identified by its H1 kinase activity. CDK2 was immunoprecipitated from equal amount of lysate in all samples (500 μg of total protein) using a specific antibody. The immunoprecipitates were assayed for its capability to phosphorylate histone H1 (with 32P-ATP) and the band for H1 protein resolved in SDS–PAGE.

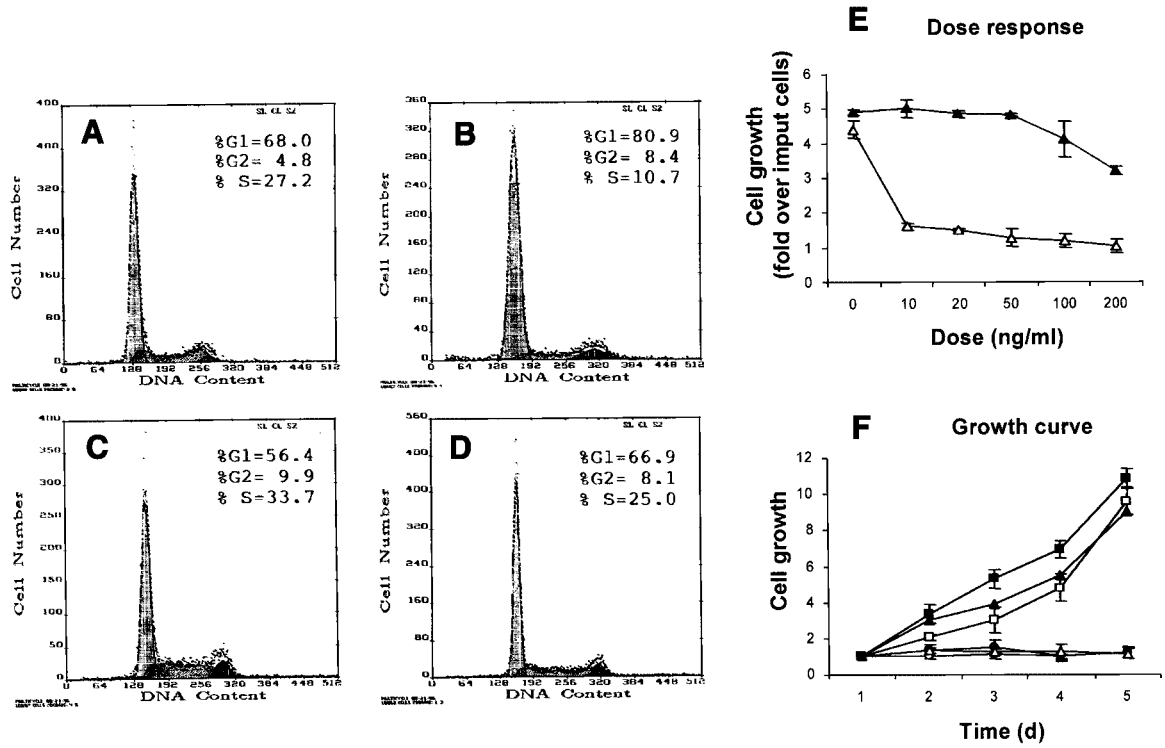

Fig. 4. Overexpression of cyclin D1 inhibits the sensitivity of A375 cells to TNF. **A–D**: Overexpression of cyclin D1 inhibits TNF-induced G1 arrest. A375 parental cells (A and B) and A375 cells ectopically overexpressing cyclin D1 (C and D) were seeded at low density (approx. 5%) in 10 cm dishes, grown until 70–80% confluence was reached and then treated (B and D) or not (A and C) with 100 ng/ml TNF for 24 h. Cell cycle phases were detected analyzing the DNA contents using FACS. **E**: Dose response effect of TNF treatment in cyclin D1 overexpressing cells. Twenty-four hours after seeding of 10^4 cells in 2.5 cm dishes the growth medium was changed (Day 0) and the indicated dose of TNF added. After 3 days, cells were fixed and stained with crystal violet. After extensive washing, crystal violet was resolubilized in 10% acetic acid and quantified at 595 nm as a relative measure of cell number. Data are presented as fold over number of imput cells at Day 0. The data are presented as mean from triplicates; bars, ± SD. A375 parental cells (△), A375 cells ectopically overexpressing cyclin D1 (▲). **F**: Time course of cyclin D1 overexpressing cells in the presence of TNF. Twenty-four hours after seeding of 10^4 cells in 2.5 cm dishes the growth medium was changed (Day 1) and 100 ng/ml of TNF added to the samples. The growth of TNF-treated (empty symbols) or untreated (close symbols) samples was stopped at the indicated time points and analyzed by staining with crystal violet. Values are expressed as the percentage of cell growth normalized to the number of imput cells (Day 1). The data are presented as mean from triplicate samples; bars, ± SD. TNF treated A375 parental cells (△), TNF treated A375 cells ectopically overexpressing cyclin D1 (□), TNF treated A375 parental cells without serum (○), TNF treated A375 cells ectopically overexpressing cyclin D1 without serum (●), untreated A375 cells (▲), untreated A375 cells ectopically overexpressing cyclin D1 (■).

Taken together, these results suggest that A375 cells overexpressing D1 cyclin are unable to arrest in response to TNF treatment. To further analyze the effect of cyclin D1 overexpression on TNF induced cell cycle arrest we performed dose response and time course studies. A375 and A375-D1 cells were cultured in the presence of different concentrations of TNF for 48 h. Subsequent analysis of the growth properties revealed a dramatically decreased sensitivity to TNF treatment of cells which overexpress cyclin D1 compared with A375 control cells (Fig. 4E).

To investigate the effect of cyclin D1 overexpression on cell growth in the presence of TNF over several days we treated A375 and A375-D1 cells with 100 ng/ml of the cytokine. A375 control cells infected with empty vector alone showed a complete growth arrest upon treatment with 100 ng/ml of TNF, whereas TNF treated A375-D1 cells which overexpress cyclin D1 grew similar to untreated controls.

However, the overexpression of cyclin D1 does not render A375 cells serum-independent for cell proliferation, even in the presence of TNF (Fig. 4F). Interestingly, we observed that cyclin D1 confers A375 cells the capability to grow at very low density forming colonies (data not shown). Our results show that cyclin D1 overexpression is sufficient to induce resistance to TNF-induced growth arrest.

Overexpression of Human Cyclin D1 is not Sufficient to Bypass Etoposide-Induced Arrest

To test whether the overexpression of cyclin D1 is able to overcome other growth arresting signals, we analyzed the effect of the DNA-damaging agent etoposide in A375 cells overexpressing cycin D1 (A375-D1). In order to confirm the activity of etoposide in A375 cells we monitored the effect on $p21^{waf1}$ protein levels, which has been shown to be induced upon etoposide treatment [Sun, 2000]. Exponentially growing cells were treated with 100 nM of etoposide for 24 h. As shown in the Figure 5A, etoposide is able to induce $p21^{waf1}$ protein in A375 cells, indicating the presence of functional p53. Accordingly, etoposide triggers the arrest of A375 cells at G2/M phase (Fig. 5C). Identical results were obtained in A375-D1 cells, indicating that cyclin D1 overexpression is not sufficient to override DNA-damage induced arrest.

In order to compare the sensitivity of A375-D1 cells and the parental A375 cells to etoposide, we performed dose-response kinetics. The cells were grown in identical conditions and treated with different concentrations of etoposide at Day 0. After 3 days of treatment, the cells were fixed and the growth measured as increase over the imput cells at Day 0. A375 and A375-D1 displayed almost identical dose-dependent growth reduction upon etoposide treatment (Fig. 5B).

This data suggest that overexpression of cyclin D1 is not sufficient to bypass etoposide-induced arrest in G2.

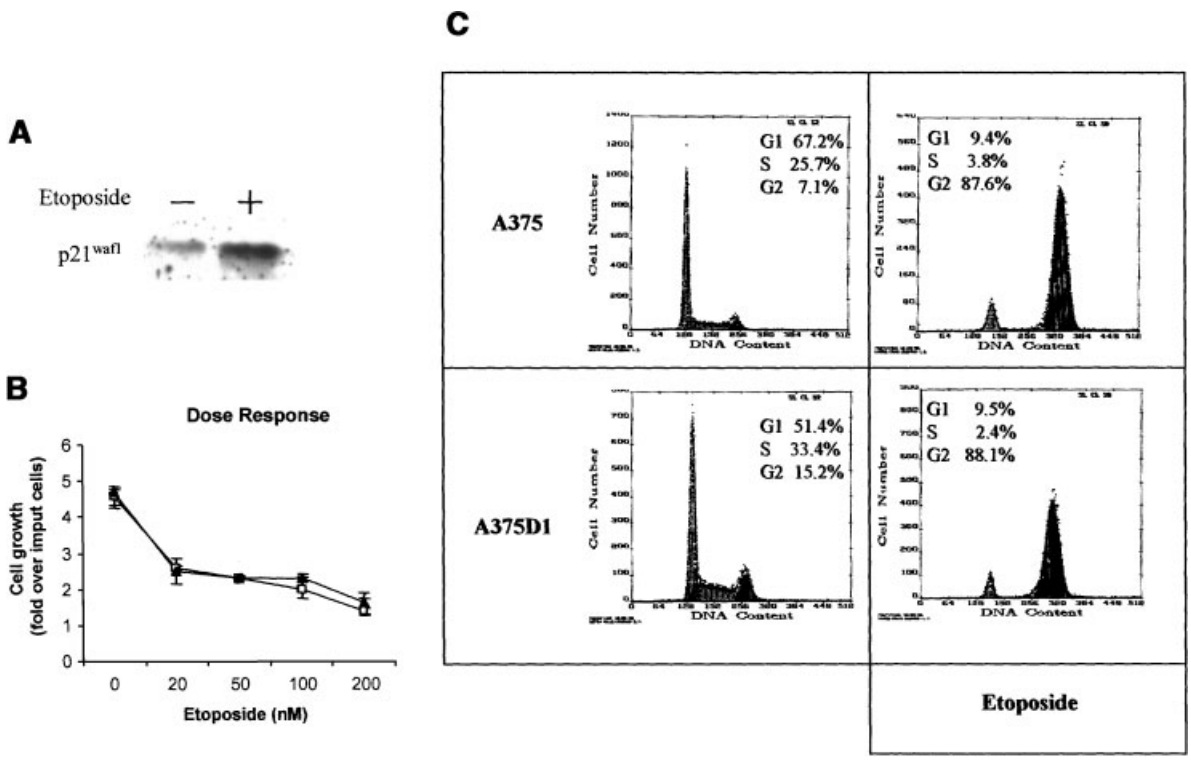

Fig. 5. Overexpression of cyclin D1 does not inhibit the sensitivity of A375 cells to etoposide. **A**: Etoposide treatment induces $p21^{waf1}$ expression. Cells were seeded at low density (approx. 5%) in 10 cm dishes, grown until 70–80% confluence was reached and then treated (+) or not (–) with 100 nM etoposide for 24 h. Cells were then lysed and 100 μg of cellular proteins resolved in SDS–PAGE. $p21^{waf1}$ were detected by immunoblotting using a specific antibody. **B**: Dose response effect of etoposite treatment in cyclin D1 overexpressing cells. Twenty-four hours after seeding of 10^4 cells in 2.5 cm dishes the growth medium was changed (Day 0) and the indicated dose of etoposide added. After 3 days cells were fixed and stained with crystal violet. After extensive washing, crystal violet was resolubilized in 10% acetic acid and quantified at 595 nm as a relative measure of cell number. Data are presented as fold over number of imput cells at Day 0. The data are presented as mean from triplicates; bars, ± SD. A375 parental cells (□), A375 cells ectopically overexpressing cyclin D1 (▲). **C**: Overexpression of cyclin D1 does not inhibit etoposide-induced G2/M arrest. A375 parental cells (A375, upper panels) and A375 cells ectopically overexpressing cyclin D1 (A375-D1, bottom panels) were seeded at low density (approx. 5%) in 10 cm dishes, grown until 70–80% confluence was reached and then treated (right panels) or not (left panels) with 100 nM etoposide for 24 h. Cell cycle phases were detected analyzing the DNA contents using FACS.

Overexpression of Cyclin D1 Increases the Amount of CDK Complexes by Posttranscriptional Mechanisms

G1 related CDK activity requires association with cyclin D to promote G_1/S transition. Mitogenic stimuli impinge on the cell cycle machinery by increasing cyclin D levels. Accordingly, altered ratio between CDKs and cyclin D affects cell cycle transition. To study the mechanisms through which overexpression of cyclin D1 might bypass the TNF-induced G1 arrest, we focused on the composition of the CDK complexes in A375-D1 cells. In these cells, overexpressed cyclin D1 increases its association to CDK2, CDK4, and CDK6 (Fig. 6A). Interestingly, total amount of CDKs also increases (Fig. 6A). Furthermore, we observed an increase of $p21^{waf1}$ bound to the immunoprecipitated CDKs even in the absence of TNF treatment (Fig. 6A). Conversely, other proteins implicated in cell cycle control such as cyclin A, cyclin E or $p27^{kip1}$ were not increased in cyclin D1 overexpressing cells (Fig. 6B). $p21^{waf1}$ has been shown to be associated with immunoprecipitates of cyclin A, cyclin D1, and cyclin E [Harper et al., 1993]. We observed increase in $p21^{waf1}$ protein in cyclin D1 overexpressing cells bound to cyclin D1, but not to cyclin A (Fig. 6C).

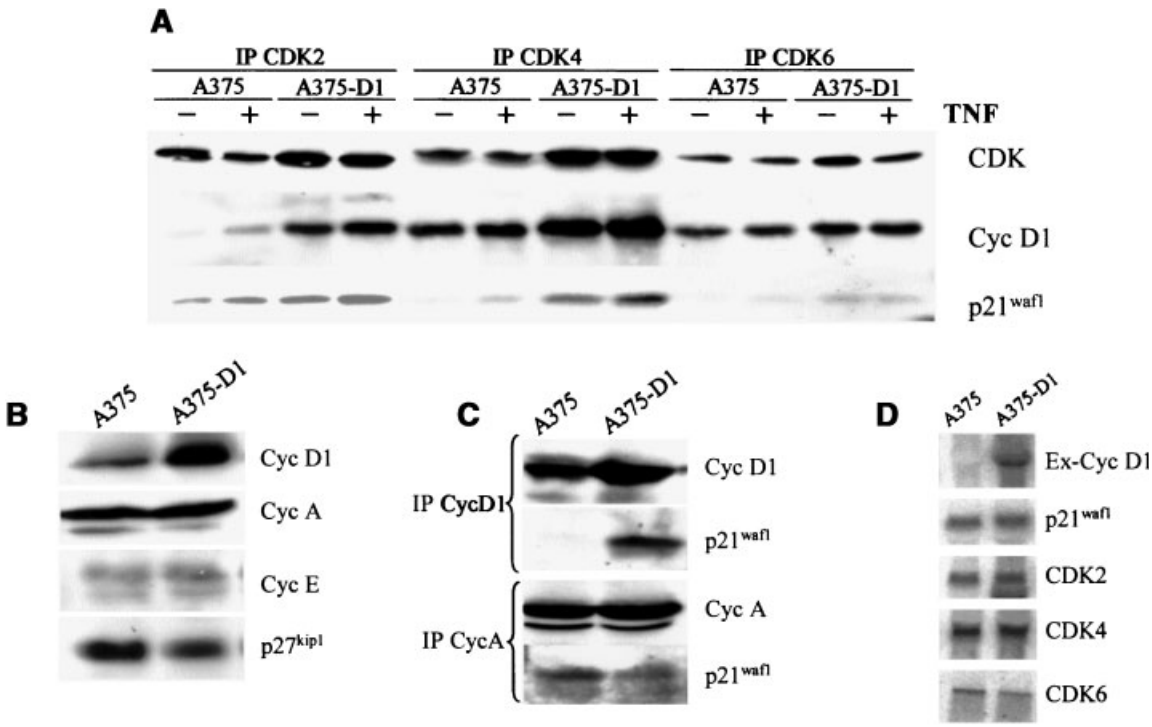

Fig. 6. Overexpression of cyclin D1 increases CDKs and $p21^{waf1}$ bound to cyclin D1 complexes. **A**: Increase of cyclin D1 bound complexes in cyclin D1 overexpressing cells. A375 parental cells (A375) and A375 cells ectopically overexpressing cyclin D1 (A375-D1) were seeded at low density (approx. 5%) in 10 cm dishes, grown until 70–80% confluence was reached and then treated (+) or not (−) with 100 ng/ml TNF for 24 h. Cells were then lysed and protein concentration valorated. CDKs were immunoprecipitated from equal amount of lysate in all samples (500 μg of total protein) using specific antibodies (IP; CDK2, CDK4, CDK6). Proteins bound to the immunoprecipitated complexes were separated by SDS–PAGE. CDKs, cyclin D1 and $p21^{waf1}$ were detected by immunoblotting using the indicated antibodies. **B**: Levels of cell cycle related proteins after TNF treatment in cyclin D1 overexpressing cells. Cells were seeded and processed as described before (A). One hundred micrograms of total protein lysate was resolved in SDS–PAGE. Each protein was detected by immunoblotting using the indicated antibodies. **C**: Overexpression of cyclin D1 increases $p21^{waf1}$ associated with cyclin D1. Cells were seeded and processed as described before (A). Cyclin D1 and cyclin A were immunoprecipitated from equal amount of lysate in all samples (500 μg of total protein) using specific antibodies (IP; CycD1, CycA). Cyclin D1, cyclin A and $p21^{waf1}$ were detected by immunoblotting using the indicated antibodies. **D**: Overexpression of cyclin D1 does not increase the level of CDKs or $p21^{waf1}$ transcripts. A375 parental cells (A375) and A375 cells ectopically overexpressing cyclin D1 (A375-D1) were seeded at low density (approx. 5%) in 10 cm dishes, grown until 70–80% confluence was reached. After mRNA extraction, 10 μg of total RNA were separated in agarose gels and transferred to nylon membranes. The northern blots were hybridized to probes specific for the indicated mRNAs.

To study whether the increase in CDKs and $p21^{waf1}$ found in cyclin D1 overexpressing cells was due to an increase in the transcriptional activity, we compared the mRNA levels of CDK2, CDK4, CDK6, and $p21^{waf1}$ in A375 and A375-D1 cells. Northern blot analysis revealed that transcript levels were not induced upon cyclin D1 overexpression pointing to posttranscriptional mechanisms which reduce protein turnover (Fig. 6D).

These results suggest that the overexpression of cyclin D1 induces the formation of more CDK complexes bound to cyclin D1. The increased number of cyclin D1 containing complexes might sequester $p21^{waf1}$ counteracting the induction of the CDK inhibitor upon TNF treatment. We hypothesize that increase in cyclin D1 proteins decreases the ratio between $p21^{waf1}$ molecules and CDK complexes blocking inhibitory activity of $p21^{waf1}$.

Antisense Fragments Against $p21^{waf1}$ Block TNF-Induced Arrest

This reasoning led us to the prediction that decrease in the levels of $p21^{waf1}$ protein should be functionally identical to cyclin D1 overexpression and produce a bypass of TNF induced cell cycle arrest. In order to confirm this hypothesis we employed an antisense approach to decrease $p21^{waf1}$ in A375 cells. Knock-down of $p21^{waf1}$ expression using antisense constructs has been reported previously [Gorospe and Holbrook, 1996; Shiohara et al., 1997; Delgado et al., 2000]. We analyzed the impact of $p21^{waf1}$ targeting on TNF-induced cell cycle arrest in A375 cells (Fig. 7). Cells were infected with retroviruses carrying $p21^{waf1}$ cDNA in antisense orientation. After selection, cells were treated for 72 h with high concentrations of TNF. The expression of the mRNA $p21^{waf1}$ antisense greatly reduced the levels of $p21^{waf1}$ protein in the cells (Fig. 7A). Physiologically, while TNF produced a dose-dependent inhibition of the growth of A375 cells in the absence of $p21^{waf1}$ antisense and complete growth inhibition was reached with 100 ng/ml of TNF, the expression of $p21^{waf1}$ antisense fragment in these cells increased the resistance to TNF treatment significantly (Fig. 7B). These results indicate that $p21^{waf1}$ is implicated in TNF-induced cell cycle arrest in A375 melanoma cells, thus supporting our hypothesis.

DISCUSSION

TNF has both in vitro and in vivo antiproliferative activities against a variety of human tumor cells. Although it has cytotoxic and cytostatic effect on malignant cells, it does not inhibit and, in fact, may stimulate the growth of diploid fibroblast and other non-transformed cell lines. A broad range of inhibitory activity has been demonstrated for recombinant TNF in vitro against human colon carcinoma, breast and cervical carcinoma, melanoma, myeloma and myeloid leukemia cell lines [Goeddel et al., 1986; Haranaka et al., 1986; Wanebo, 1989; Pusztai et al., 1993; Cheng et al., 1994; Aggarwal, 2000]. This antiproliferative activity

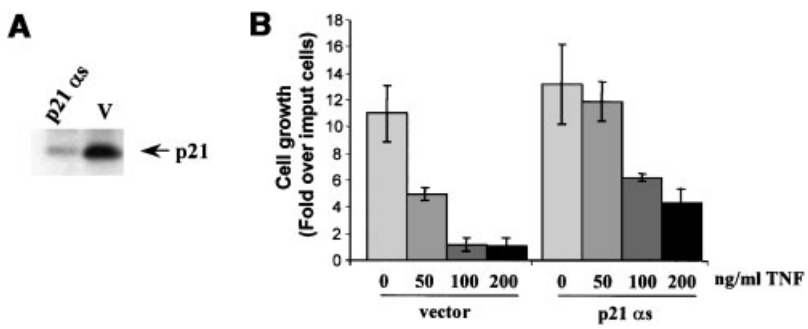

Fig. 7. Expression of a $p21^{waf1}$ mRNA antisense blocks TNF-induced cell cycle arrest. A375 cells were infected with retroviruses carrying empty vector (vector) or the $p21^{waf1}$ mRNA antisense (p21 αs) and selected with the appropriate selection drug. **A**: Once selected, cells expressing the antisense construct (p21αs) or vector alone (V) were treated for 24 h and $p21^{waf1}$ protein levels analyzed. **B**: Cells (10^4) expressing the antisense construct (p21αs) or vector alone (Vector) were seeded in 2.5 cm dishes. Twenty-four hours after seeding of the growth medium was changed (Day 0) and the indicated dose of TNF added to the samples. After 3 days, cells were fixed and stained with crystal violet. After extensive washing, crystal violet was resolubilized in 10% acetic acid and quantified at 595 nm as a relative measure of cell number. Values are expressed as fold over number of imput cells at Day 0. The data are presented as mean from triplicates; bars, ± SD.

is though to be one of the mechanisms responsible for the differential effect of macrophages in tumor cells [Urban et al., 1986; Vujanovic, 2001]. Therefore, acquired resistance to TNF may provide a mechanism for cancer cells to escape the host response. To gain insight into the possible implication of cell cycle regulators in the suppression of TNF mediated antiproliferative action, we investigated the effect of cyclin D1 overexpression in human malignant A375 melanoma cells on cellular responses to TNF treatment. In the current study, we show that overexpression of cyclin D1 is able to abrogate TNF induced cell cycle arrest which is paralleled by the sequestration of $p21^{waf1}$ by cyclin D1 containing CDK complexes.

We demonstrated that TNF treatment increased $p21^{waf1}$ expression in A375 cells as well as its association to CDK2, CDK4, and CDK6 leading to CDK inhibition and consequently to G1. Conversely, binding of $p21^{waf1}$ to PCNA, a DNA replication factor was not enhanced after TNF treatment indicating the specificity of $p21^{waf1}$ inhibitory action over CDK activity.

The amount of $p27^{kip1}$ bound to CDKs is very low and displayed no significant variation after TNF treatment. In MCF7 breast cancer cells $p27^{kip1}$ is exclusively associated with CDK4 and did not increase after TNF treatment (our unpublished data). These data suggest that $p27^{kip1}$ does not play a major role in TNF-induced cell cycle arrest. Furthermore, we did no detect alterations of other proteins implicated on G1 transition. Taken together, these results suggest that $p21^{waf1}$ is essential for the mechanims that promotes TNF-induced cell cycle arrest. This mechanistic link is further supported by our observation that the expression of antisense mRNA against $p21^{waf1}$ mitigated the antiproliferative effect of TNF.

In the work reported here, we show that stable, ectopic expression of cyclin D1 renders A375 cells resistant to TNF treatment. These cyclin D1 overexpressing cells still respond to TNF, since $p21^{waf1}$ is induced after TNF treatment. However, they grow as untreated control cells indicating that the TNF-dependent increase of $p21^{waf1}$ level is not sufficient to produce an inhibition of CDK activity in cyclin D1-overexpressing cells. The subsequent study of the CDK complexes showed that cyclin D1 overexpressing cells display elevated levels of cyclin D1-bound complexes, particularly more CDK2-cyclin D1 and CDK4-cyclin D1. The finding that overexpression of cyclin D1 blocked the TNF triggered decrease of H1 kinase activity and pRB phosphorylation provides evidence that the increased amount of cyclin D containing CDK complexes prevents inhibition of CDK2 and CDK4/6 activity.

Interestingly, we also detected an increased total amount of CDK proteins in cells stably expressing cyclin D1 compared to parental cells. However, we were unable to observe an induction of CDK2, CDK4, or CDK6 transcription. The synthesis of other proteins implicated in cell cycle control including cyclin A, cyclin E, and $p27^{kip1}$ was not increased in cyclin D1 overexpressing cells ruling out that the induction of CDK proteins is attributable to a general increased expression of cell cycle regulating proteins. Collectively, these findings suggest that overexpression of cyclin D induces a post-transcriptional mechanism which promotes the stabilization of CDK proteins.

In addition to demonstrating that the amount of CDK proteins and of cyclin D1 containing CDK complexes was elevated, we observed that the level of $p21^{waf1}$ proteins associated with CDKs, particularly with CDK2 and CDK4 increased upon cyclin D1 overexpression. $p21^{waf1}$ is a tight-binding inhibitor of CDKs and is considered as an assembly factor which stabilizes CDK complexes or as a potent inhibitor of CDK activity depending on the stoichiometry between CDK complexes and $p21^{waf1}$ proteins [LaBaer et al., 1997]. Evidence has been provided that multiple molecules of $p21^{waf1}$ are required for CDK inhibition [Zhang et al., 1994; Harper et al., 1995]. $p21^{waf1}$ was found in immunoprecipitates of CDKs, cyclin A, cyclin D1 and cyclin E [Zhang et al., 1994]. Importantly, we observed that increased binding of $p21^{waf1}$ in cyclin D1 overexpressing cells occur to cyclin D, but not to cyclin A suggesting that binding of $p21^{waf1}$ to cyclin D in A375 cells is not saturated and that there is still enough $p21^{waf1}$ protein to bind ectopically expressed cyclin D. It remains to be determined how elevated expression of cyclin D1 leads to an accumulation of CDK-associated $p21^{waf1}$. We did not observe an increase of $p21^{waf1}$ mRNA in cyclin D1 overexpressing cells indicating that the induction of $p21^{waf1}$ protein level occurs in the absence of elevated transcriptional activity.

The activities of CDKs are controlled through multiple mechanisms. The CDK subunit alone is inactive and requires both association with

a cyclin and phosphorylation on a conserved threonine residue by CDK activating kinase (CAK), for full activation. Cyclin D1 can bind CDK2, but the resulting CDK2-cyclin-D1 complex show neither H1 histone nor pRB kinase activity. The lack of kinase activity has been attributed to the fact that CDK2 associated with cyclin D1 is not phosphorylated by the CDK activating kinase CDK7-cyclin H [Higashi et al., 1996]. Our demonstration that the overexpression of cyclin D1 led to an increased association of $p21^{waf1}$ with CDK2 and cyclin D is of particular interest in the light of an inactive CDK2-cyclin-D1 complex.

The increase of non-functional CDK2-cyclin D1 may sequester $p21^{waf1}$ protein leading to a redistribution within the existing pool of CDK-CKI complexes. As a consequence, an increased number of active CDK2-cyclins A/E and CDK4/6-cyclin D1 complexes would be available. This is in agreement with our finding that H1 histone activity was increased in cyclin D1 overexpressing cells.

It is important to consider that the increased total amount of cyclin D1-CDK4/6 complexes that we described in cyclin D1 overexpressing cells might also contribute to the redistribution of $p21^{waf1}$ proteins. However D-type cyclins do not automatically assemble into complexes with CDK4 or CDK6 [Matsushine et al., 1994]. Uncomplexed D-type cyclins are rapidly degraded by PEST sequence mediated proteolysis. Interestingly, $p21^{waf1}$ has been suggested to stabilize a kinase active ternary cyclin D-CDK-$p21^{waf1}$ complex [Parry et al., 1999]. In accordance with these data the elevated cyclin D1 expression in our system and as a consequence the increased amount of CDKs may titrate $p21^{waf1}$ proteins converting it from CDK inhibitors to assembly factors that stabilize CDK activity.

The current studies may explain why overexpression of cyclin D1 suppresses TNF induced G1 cell cycle arrest. We hypothesize that the expression of exogenous cyclin D1 results in a functional reduction of $p21^{waf1}$ which in turn compensate the TNF-triggered increase of the CDK inhibitor. This assumption was substantiated by our finding that the expression of a $p21^{waf1}$ antisense fragment decreased sensitivity to TNF treatment in A375 cells. Conversely, reduction of $p21^{waf1}$ expression using antisense approach in ME180 cervical cancer cells does not inhibit the cytostatic effect of TNF [Shiohara et al., 1997] suggesting that the cytokine triggers multiple, independent signaling events to execute cell cycle arrest in these cells. Alternatively, the residual $p21^{waf1}$ in antisense expressing cells is still sufficient to promote cell cycle arrest. It will be interesting to study whether overexpression of cyclin D1 also bypass the TNF effect in ME180 cells.

In esophageal epithelial HET-1A cells overexpression of cyclin D1 led to resistance to growth inhibition by TGFβ which might be attributable to the concomitant decrease of TGFβRII. [Okamoto et al., 1994]. A recent report shows that cyclin D1 overexpression renders T-47D breast cancer cells resistant to the antiproliferative effect of the synthetic progesterone analogues progesterin, whereas overexpression of cyclin E has little effect [Musgrove et al., 2001].

Accumulation of cyclin D1 and D3 in the breast cancer cell line MCF7 is caused by a defect in the ubiquitin mediated proteolysis. In accordance with our data Russell et al. [1999] reported reduced turnover and as a consequence elevated level of $p21^{waf1}$ protein in MCF7 cells. In these cells, the inhibitory effect of $p21^{waf1}$ is overcome by the stabilization of the cyclin D1 and D3. However, MCF7 cells are sensitive to treatment with TNF [Cai et al., 1997] suggesting that in this cell line, TNF may be acting through other mechanisms than $p21^{waf1}$ increase.

Furthermore, cyclin D1 is frequently overexpressed in esophageal, breast and liver tumors. It would be of particular interest to analyze the sensitivity of cell lines derived from these tumors to TNF treatment. Several transgenic mouse models for the overexpression of D-type cyclins in different cell types have been established [Lovec et al., 1994; Wang et al., 1994; Robles et al., 1996]. The work of Wang et al. [1994] revealed that forced expression of cyclin D1 in mouse mammary gland leads to carcinoma. Analyzing the effect of TNF in this model system could provide in vivo support for the view that cyclin D1 is able to promote resistance to TNF.

In summary, we propose a stoichiomeric mechanism of cyclin D1 mediated TNF resistance which acts through the sequestration of $p21^{waf1}$ proteins by cyclin D1 containing CDK complexes abrogating the inhibitory action of the CDK inhibitor. Our results are in accordance with the hypothesis that cyclin D1 is

a critical target gene during carcinogenesis, which is capable of providing resistance to physiological growth control mechanisms acting on the G1 restriction point.

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