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# TNFα-mediated cell death is independent of *Cdc25A*

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## Abstract

**Tumor necrosis factor (TNFs) have been shown to be synthesized by ovarian carcinomas, and may therefore affect tumor cells in an autocrine manner. Therefore, we investigated the effects of recombinant TNFs on ovarian carcinoma cells N.1 and examined expression of the proto-oncogenes c-*myc* and *cdc25A* which are known to play a prominent role in apoptosis.**

**TNFα elicited apoptosis in N.1 cells within 72 h which was shown by typical morphological changes, DNA fragmentation and signature type cleavage of poly(ADP-ribose) polymerase into a 89 kDa proteolytic peptide. TNFα-induced apoptosis was accompanied by constitutive c-*Myc* expression, although the mRNA level of phosphatase *cdc25A* was suppressed within 24 h of TNFα treatment and the protein level decreased after 48 h. *Cdc25A* tyrosine phosphatase is an activator of the cdk2-cyclin E complex which allows for cell cycle progression. As expected, we found TNFα-mediated *Cdc25A* down-regulation to inhibit Cdk2 activity. *Cdc25A* suppression was related to TNFα-induced apoptosis but not to a TNFα-induced $G_0$ arrest because cyclin D1 expression was unaffected and the gene *gas6* (growth arrest specific 6) was not induced. Arresting cells by treatment with genistein prevented TNFα-triggered apoptosis and inhibited c-*myc* expression.**

**TNFα-induced apoptosis is not accompanied by cell cycle arrest which may be due to constitutive c-*Myc* expression, although *Cdc25A* and Cdk2 activity is also down-regulated. High c-*Myc* and low *Cdc25A* activity might present conflicting signals to the cell cycle machinery which are incompatible with cell survival.**

**Keywords:** apoptosis; *cdc25A*; c-*myc*; TNF

**Abbreviations:** Cdk2 (cyclin dependent kinase 2), *gas6* (growth arrest specific 6), PARP (poly[ADP-ribose] polymerase), TNFα (tumor necrosis factor alpha).

## Introduction

Ovarian carcinoma cells have been shown to produce various cytokines, growth factors and TNFs (Naylor *et al*, 1993; Wu *et al*, 1993) which may act as autocrine regulators of cell growth and death. The human ovarian carcinoma cell line N.1 also synthesizes the cytokines IL-1α, IL-6, IL-8 (Harant *et al*, 1995) and the growth factor M-CSF (Krupitza *et al*, 1995a). N.1 cells proved useful for investigating the activity of various differentiating agents, because these cells respond with morphological alterations which accompany growth inhibition (Krupitza *et al*, 1995b; Grunt *et al*, 1993). This property makes it easier to analyze novel compounds and stimuli in a first screen. Moreover, apoptosis, a type of cell death originally defined by its morphology (Kerr, 1971; Wyllie *et al*, 1980), has been investigated in N.1 cells during treatment with all-trans retinoic acid, where it was shown that apoptosis of N.1 cells correlated with c-*myc* expression (Krupitza *et al*, 1995a,c).

Since carcinomas frequently over-express the c-*Myc* transcription factor (Marcu *et al*, 1992), attention should be paid to the finding that c-*Myc* is required for some cells (e.g. Rat1, NIH3T3 and HeLa) to become responsive to TNF (Klefstrom *et al*, 1994; Janicke *et al*, 1994) and, therefore, become susceptible to TNF-treatment.

High levels of c-*Myc* also induce apoptosis of rodent fibroblasts (Evan *et al*, 1992) and myeloid cells (Askew *et al*, 1991) under sub-optimal serum conditions. Recently, Galaktionov *et al* (1996) showed that c-*Myc*-triggered apoptosis of Rat1-MycER cells was strictly dependent on the expression of *Cdc25A*, a gene regulating the cell cycle (Jinno *et al*. 1994). Moreover, *cdc25A* induction directly depended on c-*Myc* nuclear expression. These data suggest a possible link between c-*Myc*, the cell cycle and apoptosis. Therefore, we investigated the effects of recombinant TNFα on N.1 cells and studied c-*myc* and *cdc25A* gene expression, in relation to apoptosis.

We observed that TNF triggered apoptosis of N.1 cells was preceded by *Cdc25A* down-regulation despite high c-*Myc* expression. c-*Myc* is responsible for the $G_0$ to $G_1$ transition (Marcu *et al*, 1992) and thus, initiates the cell cycle. Although it was speculated whether subsequent arrest only within a particular phase of the cell cycle might induce apoptosis, the evidence suggests that rodent fibroblasts can undergo apoptosis independent of the cell cycle position (Evan *et al*, 1992).

Components of the cell cycle are tightly regulated. In vertebrates the Cdk2-cyclin E complex allows for progression through parts of $G_1$ into S-phase (for review see McLachlan *et al*, 1995). This complex can be inhibited by p27, or when tyrosine 15 of Cdk2 remains phosphorylated

(Martin-Castellanos and Moreno, 1997). De-phosphorylation of Cdk2 tyrosine 15 seems to be a prerequisite for Cdk2 activity (Morgan, 1995). Specific de-phosphorylation of Cdk2 tyrosine 15 and activation of the Cdk2-cyclin E complex is exerted by the *Cdc25A* phosphatase (Hofmann *et al*, 1994). It is therefore possible, that *cdc25A* is subjected to rigorous regulation itself, such as by posttranslational activation of *Cdc25A* by phosphorylation (Galaktionov *et al*, 1995).

In the present investigation the expression of genes, which are relevant: (i) for growth and apoptosis such as c-*Myc* (Askew *et al*, 1991; Evan *et al*, 1992; Krupitza *et al*, 1995c) and *Cdc25A* (Galaktionov *et al*, 1996), (ii) for growth arrest such as *gas6* (Schneider *et al*, 1988; Manfioletti *et al*, 1993) and (iii) for cell cycle progression and $G_0$-phase determination such as cyclinD1 (Xiong *et al*, 1991; Lukas *et al*, 1994; Resnitzky *et al*, 1994; Draetta, 1994; Pusch *et al*, 1996), was analyzed during apoptotic treatment with TNF. We also addressed the question whether *Cdc25A* down-regulation influenced Cdk2 activity during TNFα induced apoptosis, and how the downregulation of c-*Myc* affects TNFα-treated cells.

## Results

### TNFα induces apoptosis in N.1 carcinoma cells

Treatment of N.1 cells with TNFα induced apoptosis dose dependently within 72 h. Apoptosis was identified by the typical morphological characteristics such as cell shrinkage, rounding deposition of filaments and detachment from the substratum (Figure 1). DNA of TNFα treated cells was fragmented to the characteristic ladder of 180–200 base pairs and multiples thereof (Figure 2). Moreover, signature type cleavage of poly(ADP-ribose) polymerase (PARP) into a 89 kDa peptide (Tewari *et al*, 1995) showed activation of apoptosis-specific proteolysis upon exposure to TNFα (Figure 3).

### Expression of c-*myc, cdc25A,* cyclin D1, *gas6* and Cdk2 kinase activity

It has been shown previously, that TNFα-induced apoptosis required c-*Myc* expression (Klefstrom *et al*, 1994; Janike *et al*, 1994). We found that during TNFα-induced apoptosis of N.1 cells c-*Myc* protein was constitutively expressed (Figure 4). Although it has been reported by Galaktionov *et al* (1996) that *cdc25A* is a target of the transcription factor c-*Myc* in Myc-ER cells, in N.1 cells *cdc25A* transcript levels decreased after 24 h of TNFα treatment, despite unchanged c-*Myc* expression (Figure 5A). *Cdc25A* protein levels also markedly dropped after 48 h of TNFα treatment (Figure 6). Down-regulation of *Cdc25A* during TNFα-induced apoptosis was surprising since it has been shown that apoptosis of Myc-ER cells entirely depended on *Cdc25A* expression, and inhibition of *Cdc25A* expression did not permit active cell death (Galaktionov *et al*, 1996).

It is well known that *Cdc25A* regulates Cdk2 kinase activity (Morgan, 1995; Hofmann *et al*, 1994). Therefore, we measured Cdk2 kinase activity in a Cdk2-immunocomplex assay (Pusch *et al*, 1997; Perez-Roger *et al*, 1997; Rudolph *et al*, 1996) after treatment of N.1 cells with 20 ng/ml TNFα for 72 h. Cdk2 assays were repeated three times with only small differences. A representative experiment in which 20 ng/ml TNFα was applied to N.1 cells for 72 h is shown in Figure 7. When compared to untreated control cells Cdk2 activity became down-regulated during TNFα treatment

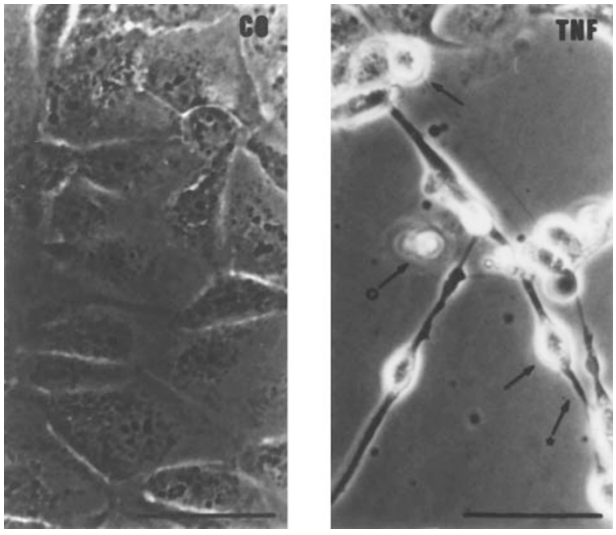


**Figure 1** Phase-contrast light microphotography of N.1 cells which were exposed to 20 ng/ml TNFα for 72 h (right panel). Untreated control cells are shown in the left panel. Scale bars (lower right corners): 50 μm

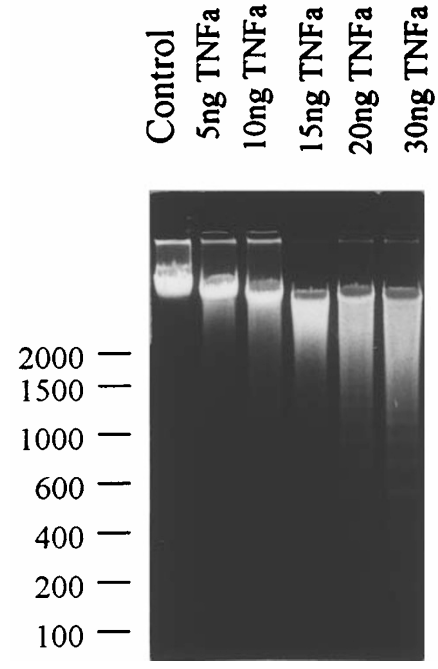


**Figure 2** DNA degradation in response to increasing concentrations of TNFα. N.1 cells were exposed to 5, 10, 15, 20, 30 ng/ml TNFα (lanes 2–6, respectively). Lane 1 shows an untreated control. The numbers to the left side of the panel indicate DNA base pairs

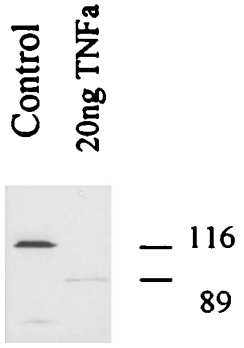


**Figure 3** Degradation of PARP during TNFα treatment. N.1 cells were treated with 20 ng/ml TNFα, protein was extracted and separated on SDS–PAGE, transferred to nitrocellulose and immunoblotted against monoclonal anti-PARP antibody C-2-10 (right lane). The lane to the left shows an untreated control. The numbers to the right side of the panel indicate kilo-Daltons

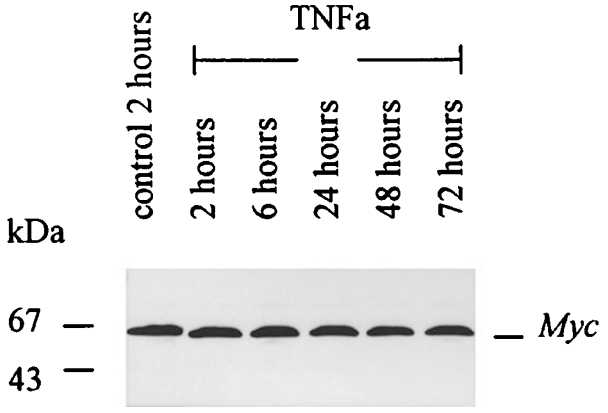


**Figure 4** Kinetics of c-*Myc* protein expression. N.1 cells were treated with 20 ng/ml TNFα for increasing periods of time (2, 6, 24, 48, 72 h); Lane 1 shows an untreated control. Protein was extracted, separated on SDS–PAGE, transferred to nitrocellulose and immunoblotted with monoclonal anti-c-*Myc* antibody

(Figure 7), comparable to the repression of *Cdc25A* (Figure 6). To examine if *Cdc25A* and Cdk2 down-regulation was due to a TNFα induced arrest in $G_0$, the expression of cyclinD1 (down-regulated in $G_0$: Motokura and Arnold, 1993; Draetta, 1994; Pusch *et al*, 1996) and *gas6* (up-regulated in $G_0$; Schneider *et al*, 1988; Manfioletti *et al*, 1993), was analyzed. Cyclin D1 expression was not inhibited nor was *gas6* expression induced after treatment with 20 ng/ml TNFα within 72 h (Figure 5B).

## Inhibition of apoptosis correlates with c-*myc* repression

When N.1 cells were treated with genistein, TNFα-induced apoptosis was abrogated (Figure 8 shows genistein-pretreated N.1 cells which were exposed to 20 ng/ml TNFα for 72 h). As a control N.1 cells were also exposed to the kinase inhibitors Ckl-7 and H-89, which did not inhibit TNFα-induced apoptosis (data not shown).

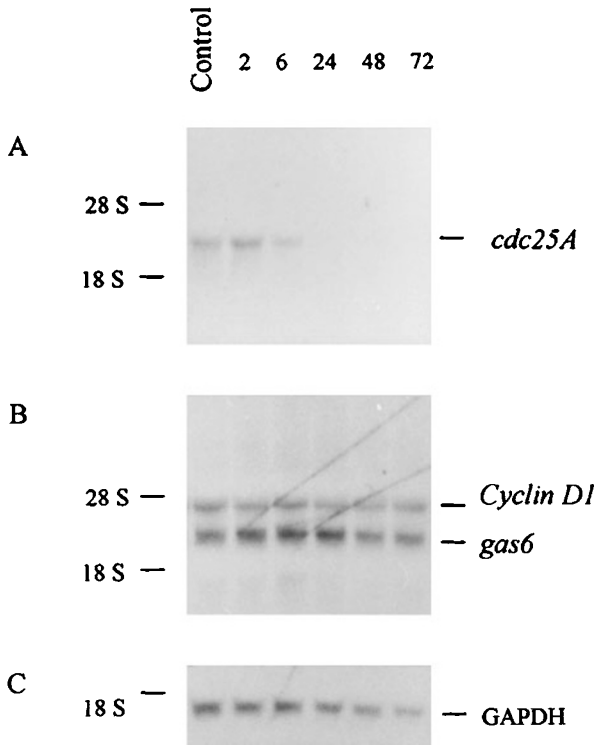


**Figure 5** Kinetics of *cdc25A*, cyclin D1 and *gas6* mRNA expression. N.1 cells were treated for 2, 6, 24, 48 and 72 h with 20 ng/ml TNFα (lanes 2–6, respectively). Lane 1 shows an untreated control. Filters were hybridized with a *cdc25A* probe (**A**), stripped and reprobed with cyclin D1 and *gas6* together (**B**), stripped and reprobed with GAPDH (**C**)

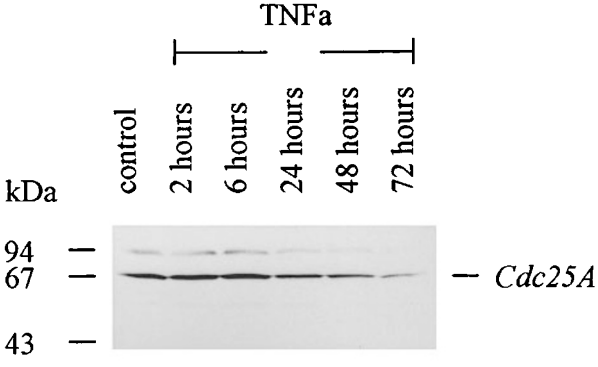


**Figure 6** Kinetics of *Cdc25A* protein expression. N.1 cells were treated for 2, 6, 24, 48 and 72 h with 20 ng/ml TNFα (lanes 2–6, respectively). Lane 1 shows an untreated control. Protein was extracted, separated on SDS–PAGE, transferred to nitrocellulose and immunoblotted with polyclonal anti-*Cdc25A* antibody

To examine whether TNFα-induced apoptosis of N.1 correlated with c-*myc* expression as previously demonstrated in Rat1, NIH3T3 and HeLa cells (Klefstrom *et al*, 1994; Janicke *et al*, 1994), the expression of c-*myc* was analyzed. In the presence of genistein the constitutive level of c-*myc* was reduced down to the limits of detection (Figure 9A, lane 3). *cdc25A* mRNA was also efficiently down-regulated upon genistein treatment


**Figure 7** Cdk2 immunocomplex kinase assay. N.1 cells were exposed to 20 ng/ml TNFα for 72 h (lane to the right). Lane 1 shows an untreated control. Protein was extracted as described under 'Materials and Methods'. Cdk2 was immuno-precipitated with monoclonal anti-Cdk2 antibody. For the kinase reaction [$^{32}P\gamma$]ATP-histone H1 was used as a target. The reaction-mix was terminated by boiling and separated on SDS–PAGE. Gels were dried and exposed to X-ray films at −80°C

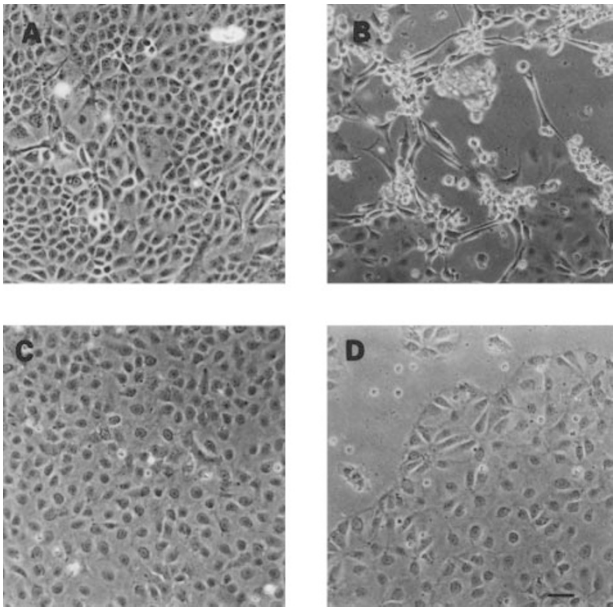


**Figure 8** Phase-contrast light microphotography of cells treated with genistein and TNFα. N.1. cells were exposed to 100 μM genistein (**C**) and to a combination of 100 μM genistein+20 ng/ml TNFα (**D**). **A** shows untreated control cells and **B** shows cells which were treated with 20 ng/ml TNFα only. The photos show cells in 100-fold magnification; the scale bar at the lower right corner of **D** indicates 50 μm

(Figure 9B; compare lane 1 *versus* lanes 3 and 4). The levels of the repressed *cdc25A* transcripts were almost the same, regardless of whether N.1 cells were exposed to TNFα+genistein (lane 4), or to genistein alone (lane 3).

In experiments in which N.1 cells were exposed to kinase inhibitors Ckl-7 and H-89 in presence of TNFα (Figure 9A,B; lanes [+]) for 15 h the epxression of c-*myc*

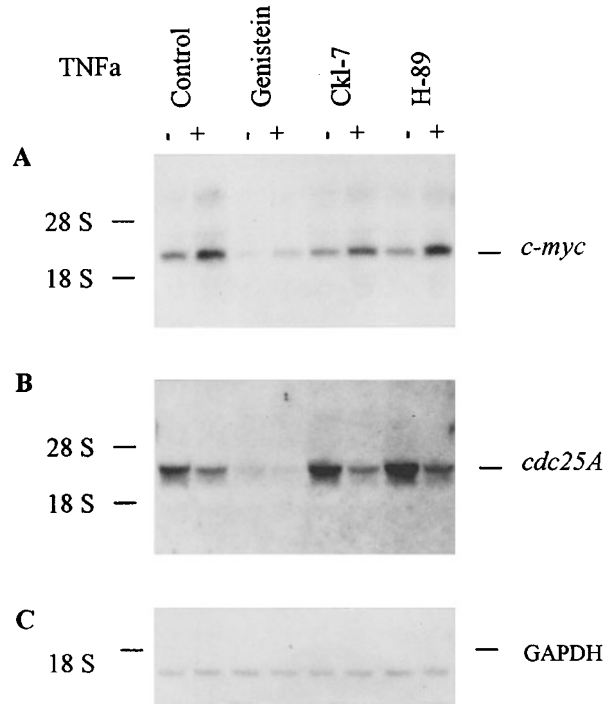


**Figure 9** Modulation of constitutive and TNFα-induced expression of c-*myc* and *cdc25A*, upon simultaneous treatment with individual inhibitors of signal transduction (**A** and **B**, respectively). During TNFα treatment (+) and under control conditions (−), inhibitors of signal transduction were co-applied. From left to right: Controls i.e. no inhibitors (lanes 1 and 2); addition of genistein, an inhibitor of tyrosine kinase (lanes 3 and 4); addition of Ckl-7, an inhibitor of casein kinase I (lanes 5 and 6); addition of H-89, an inhibitor of protein kinase A (lanes 7 and 8). After hybridization with *cdc25A* probe, filters were stripped and rehybridized with c-*myc*, and finally with GAPDH (**C**)

and *cdc25A* was found to be only marginally affected. When cells were incubated for 15 h with inhibitors alone (Figure 9A,B; lanes [−]), no significant differences of Ckl-7 and H-89 expression to control experiments were observed.

In accordance with the suggested role of high c-*myc* expression in inducing apoptosis, only treatment with genistein which inhibited TNFα-mediated apoptosis (Figure 8) also down-regulated c-*Myc*. Ckl-7 and H-89, which did not inhibit TNFα induced apoptosis had no significant effect on c-*myc* expression. This experiment further shows that down-regulation of *cdc25A* does not induce apoptosis because it is also down-regulated during genistein treatment.

## Discussion

TNFs induced apoptosis in various different cell types such as fibroblasts, hepatocytes, endothelial-, fibrosarcoma- and prostatic carcinoma cells (for review on TNFα mode of action see: Nagata, 1997; Vaux and Strasser, 1996). Here we show that N.1 ovarian carcinoma cells are also targets of TNFα-induced apoptosis.

Biochemical analysis of apoptotic TNFα-stimuli revealed that c-*Myc* expression remained high and *Cdc25A* became repressed in N.1 cells. This is contrary to the findings of

Galaktionov *et al* (1996) who showed that apoptosis in 3T3 LI cells dependend entirely on *Cdc25A*. 3T3 LI cells undergo c-*Myc* dependent apoptosis under low-serum conditions. When transfected with inducible *cdc25A* 3T3 LI cells underwent apoptosis only upon induction of *cdc25A* in low-serum containing medium. Inhibition of endogenous *cdc25A* expression by incubation of Val5 cells, which is a Myc-ER containing cell line, with an antisense *cdc25A* oligonucleotide also inhibited β-oestradiol-induced apoptosis (Galaktionov *et al*, 1996). Thus, apoptosis of Val5 cells is not only dependent on *cdc25A* expression, *cdc25A* would appear to be a functional downstream target of c-*Myc*-induced apoptosis.

When Myc-ER cells were incubated with cyclohexamide to block protein synthesis β-oestradiol-mediated translocation of Myc-ER induced endogenous *cdc25A* transcription. Thus, no intermediate protein synthesis was required for c-*Myc*-dependent *cdc25A* transcription and the conclusion was drawn that *cdc25A* in an immediate target of the transcription factor c-*Myc* (Galaktionov *et al*, 1996). Indeed, the authors also found c-*Myc* recognition sequences in introns 1 and 2 of the *cdc25A* gene.

There would appear to be other factors regulating *cdc25A* expression since in N.1 cells *cdc25A* transcription was inhibited despite constitutive c-*Myc* expression. c-*Myc*-independent regulation of *cdc25A* is supported by recent work from Pusch *et al* (1997), and also from two other groups who have reported that c-*Myc* induction did not modulate *cdc25A* expression (Vlach *et al*, 1996; Perez-Roger *et al*, 1997).

An explanation for this discrepancy could be that apoptotic stimuli might have induced either *max* and/or *mad*, thereby competing successfully for *Myc-Max* heterodimers which represent the activating transcription complex (Amati *et al*, 1993; Blackwood and Eisenman, 1991) when binding to the *cdc25A* consensus promoter sequences. *Max-Max* or *Max-Mad* complexes would compete with *Myc-Max* heterodimers for the same binding sites (Ayer *et al*, 1993), which would suppress *cdc25A* transcription. Alternatively, activity of *Cdc25A* may be regulated by phosphorylation (Galaktionov *et al*, 1995) and it is currently unresolved whether apoptosis-related regulation of *Cdc25A* involves a phosphorylated or de-phosphorylated gene product. Hypothetically overexpression of de-phosphorylated (inactive) *Cdc25A* protein might act as a dominant negative.

*Cdc25A* dephosphorylates Cdk2, thereby activating the cyclin E-Cdk2 complex (Morgan, 1995; Hofmann *et al*, 1994). As would be expected, TNFα-mediated down-regulation of *Cdc25A* was paralleled by the inhibition of Cdk2 activity as evidenced by histone H1 phosphorylation in an *in vitro* kinase assay (Pusch *et al*, 1997; Perez-Roger *et al*, 1997; Rudolph *et al*, 1996).

Despite *cdc25A* and Cdk2 down-regulation, apoptosis was independent of cell cycle effects as determined by molecular parameters such as cyclin-D1 and *gas6* expression. It was shown previously that cyclin-D1 is not expressed in $G_0$ (Motokura and Arnold, 1993; Draetta, 1994; Pusch *et al*, 1996), therefore, the consistent cyclin D1 expression observed shows that the cell cycle was not arrested in $G_0$ during TNFα-induced apoptosis. Moreover, *gas6* which becomes induced in growth arrested cells (Schneider *et al*, 1988; Manfioletti *et al*, 1993; Krupitza *et al*, 1995c) did not become up-regulated at the onset of apoptosis. Therefore, the *cdc25A* down-regulation by TNFα treatment was related to apoptosis induction but not to $G_0$ arrest.

As shown for Rat1-, NIH3T3- and HeLa cells (Klefstrom *et al*, 1994; Janicke *et al*, 1994), it seems that N.1 cells also need at least constitutive c-*Myc* levels for TNFα-induced apoptosis. Arresting cell growth by genistein abrograted TNFα-stimulated apoptosis and c-myc expression (Krupitza *et al*, 1996). c-Myc-induced progression from $G_0$ into $G_1$ with subsequent inhibition of cycle components such as *Cdc25A* and Cdk2 might create conditions incompatible with survival. To our knowledge this is the first demonstration that cells can undergo apoptosis in a *Cdc25A* independent manner.

## Materials and Methods

### Chemicals and probes

The cDNA of c-*myc* was a kind gift from Dr. Rainer deMartin, University of Vienna; that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Dr. Paul Amstad, University of Maryland; cyclin D1/prad1 from Dr. Johannes Hofmann, IMP Vienna, *cdc25A* from Dr. David Beach, Howard Hughes Medical Institute, CSH; and *gas6* from Dr. Claudio Schneider, ICGEB, Trieste. TNFα was purchased from Gibco (Paisley, UK) and TNFβ from RD-systems (Minneapolis, MN). H-89 (specific protein kinase A inhibitor, used at 1 μM final concentration) and CkI-7 (specific casein kinase 1 inhibitor, used at 10 μM final concentration) came from Seikagaku (Tokyo, Japan) and genistein (an inhibitor of tyrosine kinase activity, used at 100 μM final concentration) from Upstate Biotechnology (Lake Placid, NY), [$^{32}$Pγ]ATP was from Amersham (UK).

### Cell culture

The monoclonal human ovarian adenocarcinoma cell line N.1 which is a derivative of the heterogenous cell line HOC-7 (Buick *et al*, 1985; Grunt *et al*, 1991) was kept in αMEM supplemented with 10% fetal calf serum (Gibco, Paisley, UK) at 37°C in a humidified atmosphere containing 5% $CO_2$.

Phase contrast micrographs were taken with a Zeiss MC-80 camera connected to a Zeiss Axiovert inverted-microscope using a T-Max black and white print film (Kodak).

### Northern blot analysis

N.1 cells were grown in T-25 flasks. Experiments were terminated by discarding cell culture supernatants followed by two quick washes with ice cold PBS and subsequent lysis with RNAzol (BioTex, Houston, TX). Total RNA (30 μg/slot) was separated on a 1% agarose gel containing formaldehyde and transferred to Millipore S membranes (Millipore, Bedford, MA) by the capillary method. Biotinylated probes were allowed to hybridize to filter-bound RNA at 67°C overnight. Biotinylation procedures and filter processing were done exactly as described previously (Krupitza *et al*, 1995a). Filters were then exposed to Kodak X-ray films (Rochester, NY).

### DNA analysis

Detached cells were collected from cultures grown in T-25 flasks, centrifuged and lysed in 400 μl of a buffer containing 50 mM Tris pH 8.0, 10 mM EDTA, 0.5% sodium lauryl sarcosine. The majority of the cells, which were still attached (100% in untreated controls) were lysed in the same buffer (1200 μl). 400 μl of both types of lysates (from attached and detached cells) were treated with 2 μl RNAse A (11 U/μl, USB, Cleveland, OH) for 1 h at 37°C, followed by the addition of 10 μl of proteinase K (15 mg/ml, Boehringer Mannheim, Germany) and incubation for another 3 h at 50°C. Then equal amounts of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, Sigma) were added and DNA extracted by gentle treatment (wide-bore pipets, no vortexing). After two washes with chloroform-isoamyl alcohol (24 : 1), DNA was precipitated with alcohol, and resuspended in 30 μl TE (10 mM Tris, 1 mM EDTA, pH 7.5), 2 μl RNAse (2 U/μl). The lysates derived from attached and detached cells were pooled, the DNA content measured and equal amounts of pooled DNA subjected to electrophoretic separation on 2% agarose gels.

### Western blot analysis

Cells from treated and untreated cultures were lysed in SDS sample buffer (25 mM Tris pH 6.8, 3% SDS, 10% glycerol, 36 mM DTT, 0.925 mM EDTA). The amount of protein in each sample was calculated using the dot-metric protein assay kit (Novus Molecular Inc.) and equal amounts of protein were loaded onto 10% polyacrylamide gels. Proteins were electrophoresed at 80 V for 2–3 h and then transblotted onto nitrocellulose membranes at 100 V for 2 h (Towbin *et al*, 1979). Membranes were blocked in PBS with 0.5% skimmed milk and 0.05% Tween 20, incubated with primary antibodies (Pan-*myc*, mouse monoclonal, Genosys Biotechnologies Inc., and *Cdc25A* rabbit polyclonal, Santa Cruz Biotechnology) overnight, and with horse radish peroxidase conjugated secondary antibody for 2 h. The Amersham ECL kit was used for blot development and chemoluminescence was detected on Kodak Xomat DS films.

### Kinase assay

Protein extracts were prepared in a buffer containing 20 mM HEPES pH 7.9, 0.4 M NaCl, 2.5% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM NaF, 0.5 mM $Na_3VO_4$, 0.02 μg/ml leupeptin, 0.02 μg/ml aprotinin, 0.003 μg/ml benzamidinchloride, 0.1 μg/ml trypsin inhibitor and 0.5 mM DTT. After a freeze-thaw cycle in liquid nitrogen, cellular debris was pelleted by centrifugation and supernatant stored at −70°C (Pusch *et al*, 1997). Protein concentrations were determined using the BioRad protein assay reagent with bovine serum albumin as standard.

Cyclin-dependent kinase immunoprecipitations and Cdk-analysis was performed according to Soucek *et al* (1997). Anti Cdk2 antibody (Santa Cruz) was coupled to protein G-Sepharose beads and incubated with 30 μg protein extract for several hours at 4°C. For protein kinase assay these beads were resolved in a mixture containing 50 mM Tris pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 1 mM NaF, 80 mM ATP supplemented with radioactive ATP and 1 μg histone H1. After incubation at 37°C for 30 min, proteins were separated by electrophoresis on a 12% SDS-PAA gel. The gel was dried and exposed to X-ray film at −70°C.

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