Abstract

Interferon γ (IFNγ), a potent inhibitor of proliferation, inducer of apoptosis, and an immune modulator of mammalian cells, has been used as an anticancer agent in cancer therapy. Several molecular mechanisms, depending upon the differences in the lineage of transformed cell targets, have been elucidated for the growth inhibition or apoptosis of target cancer cells by IFNγ. However, its mechanism of action on normal cells needs to be understood from the point of view of: (i) The effect of IFNγ on non-transformed cell line and (ii) The side effect of interferon therapy on normal cells in cancer patients. Using the non-transformed cell line, human foetal epithelial cell line (WISH), our earlier studies had shown that IFNγ detains cells at a point prior to the activation step of cyclin dependent kinase 2 (CDK2) in the G1 phase of cell cycle. In the present study, we identified significant reduction in the levels and/or activity of cyclin E-CDK2, CDC25A phosphatase, cyclin H, cyclin E, cyclin D, p21 and p27. The drastic decrease in the levels and/or activity of cyclin E and/or of cyclin E-CDK2 complex might have caused growth arrest of WISH cells by IFNγ.

Keywords: Interferon γ; WISH; Cell cycle; Cyclin dependent kinase 2; Cyclin E; Proliferation; Growth inhibition

Introduction

Interferon (IFNγ) is a potent inhibitor of growth of mammalian cells (Pestka et al., 1987). Growth inhibition is found to be dependent on the activity of phosphorylated species of the latent cytoplasmic transcription factor STAT1α (Darnell et al., 1994; Bromberg et al., 1996). It is believed that the IFN effects growth arrest through cellular functions of gene products, whose synthesis is induced or repressed by the IFN. The molecular changes include reduction in the activity of cyclin dependent kinase 2 (CDK2) in mouse embryonic fibroblasts (Bromberg et al., 1996), induction of expression of CDK2 inhibitor p21 in A431 cells (Chin et al., 1996), in glioblastoma cell lines T98G, SNB-19 and U-373 (Kominsky et al., 1998), in macrophages (Xaus et al., 1999) and in breast carcinoma MCF7 cells (Gooch et al., 2000), inhibition of cyclin A gene transcription in vascular smooth muscle cells (Sibinga et al., 1999), repression of cdk2 gene expression by the interference of interferon regulatory factor 1 (IRF1) with SP1-dependent transcriptional activation of cdk2 promoter (Xie et al., 2003), hyperphosphorylation of retinoblastoma protein in mammary epithelial cells (Harvat and Jetten, 1996), and reduction in thymidine incorporation in WISH cells (Aharon et al., 2002). IFNγ has also been found to induce or sensitize target cells to apoptosis through the induction of caspase 8 by IRF1 in Ewing tumor, neuroblastoma or medulloblastoma (Fulla and Debatin, 2002) or by downregulating the levels of p21 in human hepatocellular carcinoma cells (Detjen et al., 2003). On the contrary, IFNγ induces p21, brings about growth arrest and prevents apoptosis in macrophage cells (Xaus et al., 1999).

These specific molecular changes have been suggested to be the reasons for the growth inhibition or apoptosis, or otherwise, brought about by the IFN in the respective target cell systems. The molecular changes accompanying IFNγ-induced growth arrest and apoptosis
cells per ml were seeded in a 96-well plate and exposed to 200 units per ml of human recombinant IFNγ (kind gift from Sidney Pestka and Gianni Garotta) for 72 hrs. Viable cell counts were taken once every 12 hrs or once every 24 hrs, up to 72 hrs, in triplicates using trypan blue dye exclusion method. Experimental variations were always less than 10%. For flow cytometry, WISH cells were cultured in the presence of dye exclusion method. Experimental variations were always less than 10%. For flow cytometry, WISH cells were cultured in the presence of IFNγ for 72 hrs. IFNγ was washed off and single cell suspensions were prepared. Control cells were cultured identically but without IFNγ. Cells were fixed at 72 hrs after IFN treatment, stained with propidium iodide and analysed using flow cytometry.

Western blotting
Cells were suspended in lysis buffer [50 mM Hepes-NaOH (pH 7.4), 150 mM NaCl, 1mM EDTA, 2.5 mM EGTA, 10% glycerol, 1 mM DTT, 1% Triton X-100, 25 mM sodium fluoride, 1 mM sodium vanadate, 100 mM β-glycerophosphate and protease inhibitors (1 μg/ml each final concentration), aprotonin, leupeptin and pepstatin and pefabloc (100 μg/ml final concentration)]. The cells were mildly sonicated, the lysate was centrifuged at 10,000 rpm for 10 min at 4°C and protein concentration was determined. Samples containing 100 μg equivalent total protein were fractionated on a 10% or 12% SDS-PAGE and transferred onto PVDF membrane. After blocking for 2 hrs, the membrane was incubated independently with antibodies. The membrane blots with Ponceau S stain. ImageGauge version 2.54 was used to quantitate protein bands.

Immunoprecipitation
Immunoprecipitations were performed by incubating 200 μg (in the case of CDK2 and cyclin E) or 500 μg (in the case of CDC25A) of whole cell lysate with 500 ng each of anti-CDK2 or anti-cyclin E antibodies, or 1 μg of anti-CDC25A antibody in 500 μl of the lysis buffer overnight at 4°C. Immune complexes were allowed to adsorb for 2 hrs at 4°C onto 30 μl of 50% slurry of protein A-sepharose beads, which were pre-equilibrated in 500 μl of lysis buffer for one hr at 4°C. The beads were washed thrice with lysis buffer and processed for the kinase assay or the phosphatase assay. The immunoprecipitation experiments were carried out thrice with independent sample preparations.

Cyclin dependent kinase 2 assay
Immunoprecipitated complexes using anti-Cyclin E or anti-CDK2 antibodies were washed with lysis buffer and subsequently with the reaction buffer [25 mM Hapes-NaOH (pH 7.4), 25 mM MgCl₂, 2 mM DTT, 25 mM sodium fluoride, 1 mM sodium vanadate, 100 mM β-glycerophosphate and protease inhibitor (1 μg/ml each final concentration) cocktail containing aprotonin, leupeptin and pepstatin and pefabloc (100 μg/ml final concentration)]. The beads were resuspended in 30 μl of kinase assay mixture [reaction buffer, 25 μM (β⁴⁲P) ATP and 2 μg histone H1], incubated at 37°C for 30 min and the reaction was stopped with SDS-PAGE buffer. The phosphorylated histone protein was run on 12% SDS-PAGE, gel was dried and the radiolabelled histone was quantitated by phosphorimagery analysis. The assay was repeated thrice with three independent samples.

CDC25A phosphatase assay
The CDC25A phosphatase assay was carried out as described (Jaspers and Miller, 1991). In brief, the CDC25A immunoprecipitates on protein A-sepharose beads were washed extensively with lysis buffer, followed by three washes with phosphatase buffer [25 mM Hapes-NaOH (pH 7.4), containing 5 mM EDTA, 2 mM spermidine and 2 mM DTT]. Subsequently, the beads were suspended in phosphatase assay buffer [25 mM Hapes-NaOH (pH 7.4), 50 mM 2-mercaptoethanol and 0.1 mg/ml BSA] and incubated in the presence of 10 mM para-nitrophenyl phosphate (pNPP, Sigma) at 30°C for 10 min. The reaction was stopped with 800 μl of 0.2 M NaOH and the para-nitrophenol (pNP) formed was measured at 410 nm and the total umoles of pNP formed were calculated. The assay was repeated at least thrice with three independent sample preparations.

Results
IFNγ-induced growth inhibition involved functional STAT1α
Proliferation of WISH cells was inhibited within the first 12 hrs of exposure to IFNγ (figure 1A), with accumulation of the cells in G1 phase of cell cycle (figure 1B), as reported earlier by us (Supriya et al., 1998; Vashistha et al., 2007). Induction of STAT1α, up to a level...
of 1.5-fold of the levels in the untreated cells, could be observed in WISH cells as early as 30 min post-treatment (Figure 2A), with the levels peaking to 5.6-fold at 24 hrs post-treatment. An increase in the levels of interferon regulatory factor 1 (IRF1), which is one of the primary response genes (Harada et al., 1994; Pine et al., 1990; Sims et al., 1993; Lallemand et al., 1997), peaking to 2.6-fold at 24 hrs post-treatment indicated that STAT1, which was induced in response to IFN treatment, was functionally active (Figure 2B). The same fold of IRF1 induction could also be observed as early as 9 hrs post-treatment as well (data not shown).

Levels and/or activity of cyclin E-CDK2, cyclin E and cyclin A

The levels of total CDK2, the regulatory kinase governing G1/S transition (Koff et al., 1992; Dulic et al., 1992; Tsai et al., 1993), were found to steadily decline to about 50% of the control sample by 48 hrs of IFN treatment, which then stabilised upto 72 hrs of treatment (Figure 3A and Table 1). *In vitro* assay for the activity of total CDK2 revealed a decline to 74.5% of the untreated cells at 24th hr of treatment, which steadily decreased further to 61.5% by 48th hr and to 39.7% by the end of 72 hrs of treatment (Figure 3B). Activity status of cyclin E-CDK2 complex (the active form of CDK2 during G1/S transition) in WISH cells detained by IFNγ showed severe reduction to 28.4% and further to 20% of the control sample by the end of 24th hr and 48th hr respectively, with partial recovery to only 50.2% of the control sample by the end of 72 hrs of exposure (Figure 3C).

![Figure 2: Induction of STAT1α and IRF1 in IFNγ-treated WISH cells. Western blots for STAT1α (A) and IRF1 (B) from cells that were either not treated (control) or treated with 200 U of IFNγ for 30 min, 24 hrs, 48 hrs or 72 hrs. The densitometry values (indicated below respective lanes) for the samples in the representative blots were normalised with respect to the control. Standard error values for multiple independent experiments were ± 5% and ± 8% respectively. PS: Ponceau S stained gel representing equal loading.](image)

![Figure 3: Protein and activity profiles of CDK2 in WISH cells treated with IFNγ. WISH cells were either not treated (control) or treated with 200 U of IFNγ for 24 hrs, 48 hrs or 72 hrs and the whole cell lysates were: western blotted for CDK2 (A), or assayed for total CDK2 activity (B) or cyclin E-CDK2 activity (C). The densitometry values (indicated below respective lanes) for the treated samples in the representative blots/assays were normalised with respect to the control. Standard error for multiple independent experiments was ± 10%. PS: Ponceau S stained gel representing equal loading.](image)

| Name of the cell cycle-regulated protein | Per cent level of the protein with respect to control | Duration of treatment with IFNγ (hrs) |
|----------------------------------------|-----------------|-----------------|
| CDK2                                   | 100             | 50              |
| Cyclin E                               | 50              | 10              |
| Cyclin A                               | 50              | 80              |
| Cyclin H                               | 90              | 40              |
| CDK7                                   | 80              | 120             |
| CDC25A                                 | 120             | 90              |
| p21                                    | 60              | 40              |
| p27                                    | 80              | 50              |
| Cyclin D                               | 80              | 10              |
| CDK4                                   | 90              | 90              |

The total lysates from the cells, which were treated with IFNγ for different duration, were fractionated on SDS-PAGE, western blotted for the cell cycle-regulated proteins with respective antibody and the levels of the protein in the blots were quantitated using ImageGauge version 2.54.

![Figure 4: Western blot of the protein profiles of cyclin E, cyclin A, cyclin H and CDK7. WISH cells were either not treated (control) or treated with 200 U of IFNγ for 24 hrs, 48 hrs or 72 hrs and the whole cell lysates were western blotted for: cyclin E, cyclin A, cyclin H and CDK7. The densitometry values (indicated below respective lanes) for the treated samples in the representative blots were normalised with respect to the control. Standard error values for multiple independent experiments were ± 9-13% for different proteins. PS: Ponceau S stained gel representing equal loading.](image)
Complex (Dulic et al., 1992), low levels of cyclin E component and thereby its nonavailability in required quantity to form cyclin E-CDK2 complex could be a reason for the decline of cyclin E-CDK2 activity. In fact, the levels of cyclin E showed a steep decline to 50% of the control by the 24 th hr of IFNγ treatment and thereafter to about 10% and 20% of the control sample by the 48 th and 72 nd hr of treatment respectively (Figure 4 and Table 1). Thus, one of the possible reasons for the low levels of cyclin E-CDK2 activity, which might have caused proliferation arrest of WISH cells, could be the absence of required quantity of cyclin E to form an active cyclin E-CDK2 complex to enable G1/S transition.

Further, cyclin A is required for the onset of DNA replication in early S phase (Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992; Rosenblatt et al., 1992) through the formation of active cyclin A-CDK2 complex (Connell-Crowley et al., 1993). Therefore, lack of required quantity of cyclin A would also induce detention of cells prior to DNA synthesis. In WISH cells exposed to IFNγ, the levels of cyclin A initially declined to 50% of the control cells by the 24 th hr of treatment (Figure 4 and Table 1), by which time growth inhibition had already occurred as revealed by viable cell count assay (Figure 1A). However, unlike cyclin E, subsequently it showed recovery to 80% of the control by the 48 th hr of exposure, but followed by a decline to 30% of the control by the 72 nd hr of exposure (Figure 4 and Table 1). At present, we do not have an explanation for the repeatedly and consistently observed cyclic pattern of cyclin-A levels in IFNγ-treated WISH cells.

Levels and/or activity of CAKII and CDC25A

One of the other possibilities for the low activity of cyclin E-CDK2 could be the absence of activating phosphorylation on T160 residue of CDK2 by CDK2 activating kinase II (CAKII) (Gu et al., 1992), which is a complex of CDK7 and cyclin H (Poon et al., 1993; Fisher and Morgan, 1994; Kim et al., 1996). Nevertheless, the levels of cyclin H and CDK7 were largely unaffected up to 24 hrs of IFN treatment (Figure 4 and Table 1), by which time the cells had already undergone cell cycle arrest (12 th hr itself; see Figure 1A), thereby ruling out the immediate contribution of cyclin H-CDK7 complex in compromising the activity of CDK2. Yet another possibility for the reduced activity of CDK2 could be the lack of activation of CDK2, owing to the lack of dephosphorylation of T14 and Y15 residues by CDC25A phosphatase (Gu et al., 1992; Gabrielli et al., 1992). The activation of CDC25A phosphatase itself is regulated by a cyclin E-CDK2 dependent phosphorylation during the G1/S transition, as part of an auto-amplification loop (Hoffmann et al., 1994). Thus, it is likely that CDC25A phosphatase itself might be inactive in IFNγ-arrested WISH cells that exhibited low levels of cyclin E-CDK2 activity.

However, CDC25A protein levels were maintained up to 48 hrs (Figure 5A and Table 1). Notably, the activity of CDC25A also did not show reduction at 24 th hr, by which time (12 hrs before itself) the cells were already growth-inhibited. In fact, it showed a marginal but repeatedly consistent increase of about 30% over that of control. Subsequently, the activity steadily declined to 46% and further to 29% of the control by the 48 th and 72 nd hr of treatment respectively (Figure 5B and Table 1). Thus, the protein levels and activity of CDC25A were maintained, if not showed a slight consistent increase at 24 th hr, when cyclin E-CDK2 activity had already decreased to 28.4%. It suggested that CDC25A phosphatase activity was not affected at least till 24 th hr by the decline of activity of cyclin E-CDK2 in IFNγ-treated WISH cells. However, one of the possibilities for the decrease in the activity of CDC25A at 48 th and 72 nd hrs of exposure could be lack of activation of the phosphatase. This might be due to the lack of

Since cyclin E is required for the formation of active cyclin E-CDK2 complex (Dulic et al., 1992), low levels of cyclin E component and
activating phosphorylation on the phosphatase by cyclin E-CDK2 as part of the autoamplification loop (Hoffmann et al., 1994), owing to insufficient levels of active cyclin E-CDK2 at these time points. The steep decline in cyclin E-CDK2 activity to 10-20% subsequent to 24th and 48th hr of exposure supports this possibility.

Levels of p21, p27, cyclin D and CDK4

The CDK inhibitors p21 and p27 are known to inhibit CDK2 activity of cyclin E-CDK2 complex by physical association with the complex (Aprilekova et al., 1995; Sheaff et al., 1997; Morisaki et al., 1997; Sherr and Roberts, 1999; Xu et al., 1999). Proliferation arrest induced by IFNγ has often been accompanied by an increase in the levels of p21, with concomitant reduction in CDK2 activity in different cell systems (Chin et al., 1996; Kominsky et al., 1998; Xaus et al., 1999; Gooch et al., 2000). However, contrary to these findings, the levels of p21 as well as of p27 were found to be low in IFNγ-induced cell cycle arrested WISH cells. Between the two CDK inhibitors, p21 was more severely affected with its levels dropping to 60%, to 40% and on to 10% of the control sample at 24th, 48th and 72nd hr of treatment respectively (Figure 6 and Table 1). The levels of p27 reduced to 80%, to 50% and to 2% at these time points of IFN treatment (Figure 6 and Table 1). Absence of any up regulation in the levels of p21 and p27 suggested that these CDK1 proteins have not contributed to the reduced cyclin E-CDK2 activity observed in the cell cycle arrest induced by IFNγ in WISH cells.

Since active cyclin D-CDK4 complex is required for G1 progression (Baldin et al., 1993; Tam et al., 1994; Sherr et al., 1994), reduction in the levels and/or activity of cyclin D-CDK4, if induced by IFNγ, could effect cell cycle arrest in G1 phase. However, the levels of cyclin D were maintained at about 80% of the control at 24th hr of exposure to the IFN, subsequently declining to 10% of the control by the 48th hr and to 20% of the control by the 72nd hr of treatment (Figure 6 and Table 1). On the contrary, levels of the cognate kinase CDK4 remained unaffected throughout the 72 hrs of exposure to IFNγ (Figure 6 and Table 1).

Discussion

In the present study, among the panel of molecular changes observed in the IFNγ-induced growth-arrested WISH cells, the most striking one was the dramatic decline in the activity of cyclin E-CDK2, with the concomitant reduction in cyclin E levels to 50% by the 24th hr and eventually to barely detectable levels. This indicated the possible non-availability of cyclin E for the formation of an active cyclin E-CDK2 complex, thereby causing cell cycle arrest. However, cyclin E synthesis is known to occur only during late G1 and prior to which cyclin E levels are undetectably low (Ohsubo et al., 1995). Nevertheless, in silico analysis of about 5 kb region upstream of cyclin E open reading frame revealed the presence of a putative consensus IRF1 binding sequence ATATAAGGAAACT (the consensus sequence being GAGGCGGGGC/T C/C) at nucleotide position -2963 to -2951. Therefore, although the consensus IRF1 binding sequence is far upstream of cyclin E open reading frame, an IRF1-dependent repression of cyclin E promoter cannot be ruled out. Induction of IRF1 transfectants of NIH 3T3 fibroblast cells was found to downregulate cdk2 gene expression by interfering with Sp1-dependent transcriptional activation of cdk2 promoter, resulting in severe decline of CDK2 levels within 8 hrs of induction (Xie et al., 2003). In similar lines, an IRF1-mediated interference of Sp1-dependent transcription of cyclin E promoter, as observed in the case of transcriptional downregulation of cdk2 promoter by IFNγ (Xie et al., 2003), cannot also be ruled out as Sp1 binding sites (the consensus sequence being AGGGCGGGGC, Shiffman et al., 1996) are present at nucleotide positions -491 to -482 (GGGGCGGGGGT), -865 to -856 (GGGGCGGGGGG) and -1264 to 1255 (GGGGCGGGGA). Although CDK2 levels were stable at 24th hr and declined only at 48th hr of exposure, in spite of IRF1 levels peaking to 2.6-fold as early as 9th hr of exposure to the IFN (data not shown) and being maintained at the level upto 24th hr, it is possible that the 50% levels of CDK2 may not be sufficient to take cell cycle progression forward, owing to the low levels of cyclin E. Above all, the activity of cyclin E-CDK2 complex was also found to decrease steadily in response to IFNγ treatment.

The pattern of gradual decline in the total CDK2 activity in growth-arrested WISH cells (74.5% at 24th hr, 61.5% at 48th hr and 39.7% at 72 hr post-treatment) is in contrast to the sudden inhibition of total CDK2 activity, as early as within 6 hrs of exposure to the IFNγ, in growth-arrested mouse embryonic fibroblast cell line and Stat1 transfectant of Stat1-null U3A cells (Bromberg et al., 1996) and glioblastoma cell lines T98G, SNB-19 and U-373 (Kominsky et al., 1998). However, in these cell lines, in spite of the reduction in total CDK2 activity, the levels of CDK2 were found to remain unaffected. In growth-arrested WISH cells, in spite of the decline in cyclin E levels, the maintenance of total CDK2 activity at 74.5% and 61.5% at 24th and 48th hrs of exposure might be due to a probable association of CDK2 with cyclin A, whose levels were maintained at 50% and 80% at 24th and 48th hrs of exposure. However, we have not verified this possibility. However, it is possible that the sharp decline in its levels within 24 hrs post-IFNγ treatment might be low enough to maintain G1/S arrest of WISH cells induced by low activity of cyclin E-CDK2. In contrast to the slow turnover of cyclin A in WISH cells, IFNγ treatment had been found to drastically inhibit cyclin A transcription by as early as 12-24 hrs of exposure in vascular smooth muscle cells (Sibinga et al., 1999) and human mammary epithelial cells (Harvat and Jetten, 1996). However, unlike in WISH cells, the levels of CDK2 and cyclin E remained more or less unaffected in these cells.

Downregulation of cyclin D expression has been found to occur in murine bone marrow-derived macrophages within 24 hrs of exposure to IFNγ (Cocks et al., 1992). However, in contrast to this observation, cyclin D was found upregulated to a level of 3.8-fold in vascular smooth muscle cells exposed to IFNγ (Sibinga et al., 1999). Unlike in these cell systems, in growth-arrested WISH cells, the levels of cyclin D (and of CDK4) were maintained at the 24th hr of exposure, by which time the cells had already suffered growth arrest by the 12th hr of exposure. The decline in cyclin D levels at the 48th and 72nd hrs of exposure might possibly be reflective of the cyclin turnover, as the cyclin was no more required for G1 progression owing to inhibition of cell cycle progression. This suggested that involvement of cyclin D and/or of cyclin D-CDK4 complex was unlikely in the cell cycle arrest of WISH cells by IFNγ. Therefore, the activity status of cyclin D-CDK4 complex was not examined.

IFNγ-specific induction of IRF1 has also been found to favour apoptosis through the induction of caspase 8 by IFNγ (Fulda and Debatin, 2002). In addition, reduced levels of p21 have been reported to lead to apoptosis or sensitise human hepatocellular carcinoma cells (Detjen et al., 2003), human breast cancer (Fan et al., 2003) and human colon cancer (Tian et al., 2000) towards apoptosis. However, contrary to these studies, in growth-arrested WISH cells, neither the sharp decline in p21 levels (to 10%) nor the 2.6-fold increase in IRF1 levels triggered apoptosis even after 72 hrs of treatment with IFNγ (data not shown). In our studies, the phosphorylation status of Rb was
not found to exhibit a specific pattern (data not shown) disallowing us to comment on any specific contribution of Rb to IFN-mediated cell cycle arrest of WISH cells.

Thus, taken together, considering the presence of IRF1 binding consensus site upstream of cyclin E gene, Sp1 binding site upstream of cdk2 gene for interference by IRF1, steady decrease in the steady state levels of cyclin E (50%, 10% and 20%) and of CDK2 (100%, 50% and 50%) (Table 1) and steady decrease in the activity of cyclin E-CDK2 complex, it may be suggested that growth arrest of WISH cells occurred due to downregulation of cyclin E and cdk2 genes. The low levels of cyclin E and CDK2 would have resulted in the low levels of cyclin E-CDK2 complex and thereby low levels of active cyclin E-CDK2 complex, leading to arrest of cell cycle progression. Since the active cyclin E-CDK2 complex is required for progression of cell cycle through G1/S boundary, these observations are in concurrence with our earlier findings that IFNα detains WISH cells at G1/S boundary by effecting growth-arrest in late G1 phase (Supriya et al., 1998; Vashistha et al., 2007). The molecular mechanisms that lead to the down regulation of the levels of cyclin E require further detailed investigations.

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