Loss of STAT1 expression confers resistance to IFN-γ-induced apoptosis in ME180 cells

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Abstract Interferon gamma (IFN-γ) induces apoptosis in many tumor cell lines and sensitizes tumor cells to apoptosis by tumor necrosis factor family members. IFN-γ induces the expression of many early response genes such as interferon regulatory factor-1 (IRF-1) by activation of signal transducer and activator of transcription (STAT) factor proteins. We found that ME180 cells became resistant to IFN-γ-induced cell death after 4–5 passages in culture. These resistant cells were characterized by a loss of STAT1 expression and a loss of inducible IRF-1 expression. We describe for the first time the emergence of a STAT1-deficient ME180 cell line.

Key words: Apoptosis; Interferon regulatory factor-1; Signal transducer and activator of transcription-1; ME180; Resistance

1. Introduction

Interferon gamma (IFN-γ) is a cytokine that plays an important role in immune surveillance and host defense against infection. Recent studies show that IFN-γ induces apoptosis in many tumor cell lines [1–3]. IFN-γ also sensitizes tumor cell lines to apoptosis by members of the tumor necrosis factor (TNF) family such as TNF, Fas and TNF-related apoptosis-inducing ligand (TRAIL), also known as Apo2L [3–6]. Several candidate genes have been identified which may mediate IFN-γ-induced apoptosis such as interleukin-1β-converting enzyme (ICE) (caspase-1) and DAP-kinase [2,6,7]. Overexpression of DAP-kinase in HeLa cells, for example, causes apoptosis and reduced DAP-kinase levels in some solid tumors inhibit apoptosis and enhance tumor metastasis [7,8]. Overexpression of caspase-1 has also been shown to induce apoptosis [2,6]. It is not clear, however, if caspase-1 alone directly activates an apoptotic signaling pathway.

IFN-γ induces the expression of many early response genes by tyrosine phosphorylation of Jak kinases and signal transducer and activator of transcription (STAT) transcription factor proteins [9,10]. The Jak/STAT pathway is responsible for initial transmission of the IFN-γ signal to the nucleus but proper regulation of the many genes induced by IFN-γ involves other transcription factors such as interferon regulatory factor-1 (IRF-1) [11]. Induction of IRF-1 is dependent on the STAT1 protein [12,13]. Several studies have shown that IRF-1 is not only a transcriptional activator but it is also a critical mediator of radiation- and chemotherapy-induced apoptosis and cell cycle arrest [1,14,15]. IRF-1, for example, cooperates with p53 to induce p21 and mediate cell cycle arrest in response to DNA damage [16].

Recent studies have shown that STAT1 plays a key role in induction of apoptosis by TNF and IFN-γ. For example, Kumar et al. [17] showed that cells that lack STAT1 are resistant to apoptosis because of low constitutive levels of caspases. Chin et al. [2] showed that activation of the STAT signaling pathways in tumor cells causes expression of caspase-1 and apoptosis. Expression of ICE was shown to be essential for IFN-γ-induced apoptosis. These studies suggest a role for STAT1 in coordinating caspase-dependent apoptotic pathways mediated by IFN-γ and TNF.

There are some studies, however, which show that IFN-γ induces cell death by a non-caspase-mediated mechanism [18,19]. For example, in the ME180 cervical carcinoma cell line and A549 non-small cell lung cancer cell line, studies show that IFN-γ induces cell death by depletion of tryptophan and intracellular nicotinamide adenine dinucleotide [18,19]. Aune and Pogue [18] showed that IFN-γ does not induce DNA strand breaks in ME180 and A549 cells which suggests a non-caspase-mediated cell death.

We found, however, that cell death induced by IFN-γ in ME180 cells is mediated by caspases because cell death was almost completely blocked by z-VAD.fmk, a tetrapeptide caspase inhibitor. We also observed that ME180 cells become resistant to the cytotoxic effects of IFN-γ after 4–5 passages in culture. In comparing ME180 cells that became resistant to IFN-γ (ME180R) to ME180 sensitive cells (ME180S), we found that STAT1 protein expression was lost in ME180R cells and IRF-1 was not induced in response to IFN-γ. Overexpression of IRF-1 induced cell death in ME180R cells so that loss of sensitivity to IFN-γ likely results from a loss of STAT1 and inducible IRF-1 expression in ME180R cells. This is the first example of the emergence in tissue culture of a STAT1-deficient cell line.

2. Materials and methods

2.1. Cell culture

The ME180 cell line was purchased from ATCC (Rockville, MD). ME180 cells were cultured in either McCoy's medium with 10% fetal calf serum or RPMI 1640 with 5% fetal calf serum and 5% newborn calf serum supplemented with L-glutamine, penicillin and streptomycin.

2.2. Cell viability assay

Cell viability was measured by an MTT assay. Briefly, untreated cells or cells treated with IFN-γ in a 96-well plate were harvested at the indicated times followed by the addition of 3-(4,5-dimethylthiazol-

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Abbreviations: IFN-γ, interferon gamma; ICE, interleukin-1β-converting enzyme; IRF-1, interferon regulatory factor-1; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor

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2-yl)-2,5-diphenyltetrazolium bromide (MTT) and then cells were solubilized with 0.1 N acidified CH_3Cl-HCl. The 96-well plate was read at a wavelength of 590 nm on an iEMS Labsystems plate reader. The effect of IRF-1 on cell viability in ME180R cells was examined by transient transfection of ME180R cells by lipofectamine in a 12-well culture dish (5×10^4 cells/well) with 100 ng of the reporter plasmid pcDNA3-β-galactosidase plus 0–400 ng of pCEP4-IRF-1. The total amount of transfected DNA was kept constant at 0.5 μg with the addition of the parental pCEP4 vector. Non-apoptotic or apoptotic cells were detected by staining with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside. Percent viable cells represents the number of blue cells for each condition/total number of blue cells in control (pCEP4). Data are the mean values from three experiments (mean ± S.D.).

2.3. Cytokines, antibodies, and inhibitors

IFN-γ with a specific activity of 1×10^7 IU/ ml was purchased from Biosource Int. (Camarillo, CA). z-VAD.fmk was purchased from Enzyme Systems Products (Dublin, CA). Anti-IRF-1 polyclonal rabbit antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ICE polyclonal antibodies was purchased from UBI Systems (Lake Placid, NY). Caspase-3 and STAT1 monoclonal antibodies were purchased from Transduction laboratories (Lexington, KY). A polyclonal rabbit ERK2 antibody was from Calbiochem (La Jolla, CA).

2.4. Northern blot analysis

RNA was harvested from A549 cells with a RNA STAT-60, a solution containing guanidine isothiocyanate and phenol (Tel-Test 'B', Friendswood, TX) and Northern blot analysis was done as previously described [3]. The STAT1 cDNA was obtained from James Darnell (Rockefeller University, New York, NY). The IRF-1 human cDNA was generated as previously described [3].

2.5. Antibodies and immunoblotting

Western blot analysis was done essentially as described [20]. Briefly, ME180 cells were treated with IFN-γ for the indicated times and then were lysed in a boiling solution containing 1% SDS, 1 mM sodium vanadate and 10 mM Tris-HCl pH 7.4. Samples were centrifuged for 5 min to remove insoluble material followed by measurement of protein concentration by the Bradford method (Bio-Rad Laboratories, Hercules, CA). Samples containing equal protein concentrations were denatured by boiling and analyzed by SDS-PAGE, and then transferred to nitrocellulose. The blot was then placed in blocking buffer containing 1% milk, 1% BSA, 10 mM Tris-HCl pH 7.5, 100 mM NaCl, and 0.1% Tween 20 for 1 h at room temperature or overnight at 4°C. The blot was then incubated in blocking buffer with individual antibodies in a solution which contained 10 mM Tris-HCl pH 7.5, 100 mM NaCl, and 0.1% Tween 20 followed by incubation in blocking buffer containing a horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (Caltag Laboratories, San Francisco, CA) at a dilution of 1:1500 and detected by ECL (Amersham, Arlington Heights, IL) followed by autoradiography.

3. Results and discussion

We have examined multiple tumor cell lines for sensitivity to IFN-γ-induced cell death. Several studies have suggested that IFN-γ-induced cell death is mediated by caspase-1 [1,6,21]. There is one recent study that showed IFN-γ induces bak mRNA in HT-29 colon cancer cells along with several caspases [16]. We observed that IFN-γ induces cell death in approximately 50% of cells in the ME180 cervical carcinoma cell line (Fig. 1A). IFN-γ-induced cell death was blocked by z-VAD.fmk, a tetrapeptide caspase inhibitor (Fig. 1A). Cell death induced by IFN-γ was confirmed as apoptotic in ME180S cells by annexin staining (data not shown). Interestingly, the ME180 cell line became almost completely resistant to IFN-γ-induced cell death after 4–5 passages in culture (Fig. 1B). Simply culturing the ME180 cells in serum-containing medium induced resistance to IFN-γ-induced cell death. The use of different medium preparations, McCoy versus RPMI,

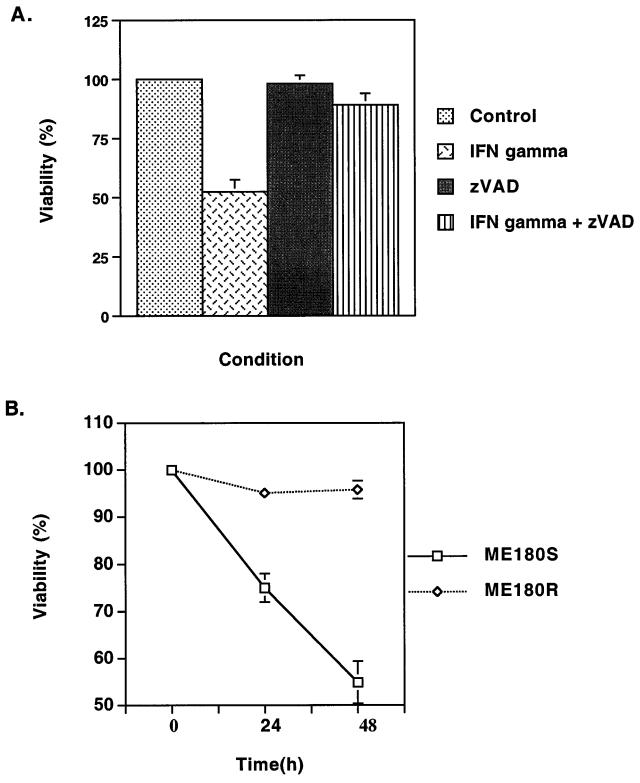

Fig. 1. Loss of IFN-γ-induced apoptosis in ME180 cells. A: MTT assay shows % viable ME180 cells after treatment with IFN-γ (250 U/ml) for 48 h in the presence or absence of z-VAD.fmk (40 μM). Cells were pretreated with z-VAD.fmk for 2 h prior to the addition of IFN-γ. B: MTT assay of ME180S and ME180R cells exposed to IFN-γ for 48 h. Data represent means of three experiments ± S.D.

or reduced fetal calf serum concentrations did not affect the emergence of IFN-γ-resistant ME180 cells. The cells had not been exposed to IFN-γ and several different aliquots from ATCC showed the same emergence of resistance.

To characterize the defect in ME180R cells which abrogates IFN-γ-induced cell death we first examined expression of IRF-1, a critical regulator of IFN-γ-induced apoptosis. Basal levels of IRF-1 mRNA were similar in both ME180S and ME180R cells but IRF-1 mRNA was induced in response to IFN-γ only in ME180S cells (Fig. 2A). We then examined IRF-1 protein in both sensitive and resistant cells and inducible IRF-1 was detected only in ME180S cells (Fig. 2B). These results show that expression of inducible IRF-1 mRNA and protein is lost in ME180R cells.

Expression of IRF-1 has been shown to be dependent on STAT1, so we examined STAT1 expression in both sensitive and resistant ME180 cells. Two forms of STAT1 mRNA were observed by Northern blot both of which were reduced at baseline and in response to IFN-γ in ME180R cells (Fig. 3A). STAT1 protein was induced by IFN-γ in ME180S cells but there was almost no basal or inducible STAT1 expression in ME180R cells (Fig. 3B). We did observe barely detectable levels of STAT1 protein in ME180R cells with overexposure of the Western blot (data not shown). These results suggest that loss of inducible IRF-1 is secondary to a defect in STAT1 expression in ME180R cells.

A central role for IRF-1 in mediating cell death responses to chemotherapy and radiation has been described, so we

hypothesized that loss of IRF-1 in ME180R cells was responsible for the defect in IFN-γ-induced apoptosis. To examine this possibility we overexpressed IRF-1 in ME180R cells and we observed an over 50% reduction in cell viability in ME180R cells in comparison to a transfected vector control (Fig. 4). These data show that we can reconstitute a cell death response in ME180R cells with overexpression of IRF-1. Interestingly, ME180R but not ME180S cells were also resistant to TNF-α-induced apoptosis but they remained sensitive to cell death induced by TRAIL, also known as Apo2L (K.Y. Lee and G.D. Rosen, unpublished observations).

Recent studies suggest a key role for STAT1 in coordinating apoptotic responses to IFN-γ and TNF [2,17]. Additionally, loss of STAT1 expression appears to result in decreased expression of caspase-1 and caspase-3 [17]. Also, activation of the STAT signaling pathway and ICE gene expression appears to be necessary for IFN-γ-induced apoptosis [2]. We observed that STAT1 expression was almost completely absent in ME180R cells (Fig. 3). We hypothesized, therefore, that expression of caspase-1 and caspase-3 would be decreased in ME180R cells in comparison to ME180S cells. However, we observed significant levels of caspase-1 and caspase-3 in both ME180S and ME180R cell lines and the levels were similar

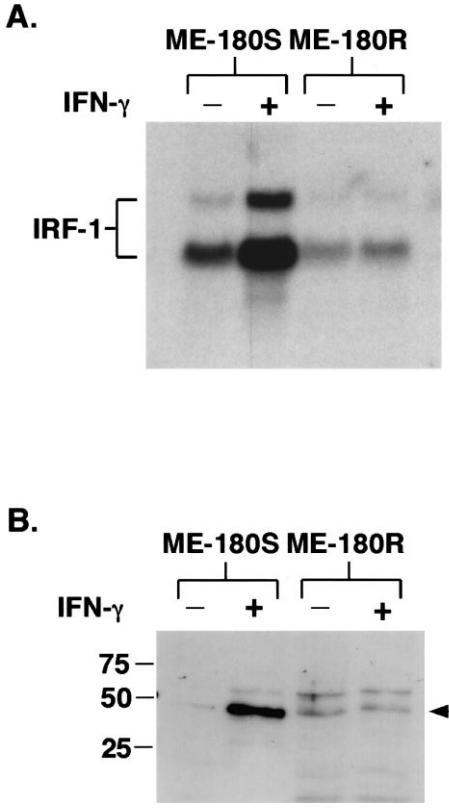

Fig. 2. Inducible expression of IRF-1 is lost in ME180R cells. A: ME180S and ME180R cells were treated for 6 h with IFN-γ and then RNA was extracted for Northern blot analysis of IRF-1. Each lane contains 10 μg of total RNA and the blot was probed with a ^{32}P-labeled 400 bp IRF-1 cDNA. The lower band corresponds to a size of 2.0 kb which is the predicted size of IRF-1. The upper band migrates at approximately 3.0 kb and likely represents an alternatively spliced IRF-1 cDNA. B: Western blot analysis of ME180S and ME180R cells treated for 8 h with IFN-γ. Cellular protein was harvested followed by loading of equal amounts of protein on an SDS-PAGE gel which was probed with an anti-IRF-1 polyclonal antibody after transfer to nitrocellulose.

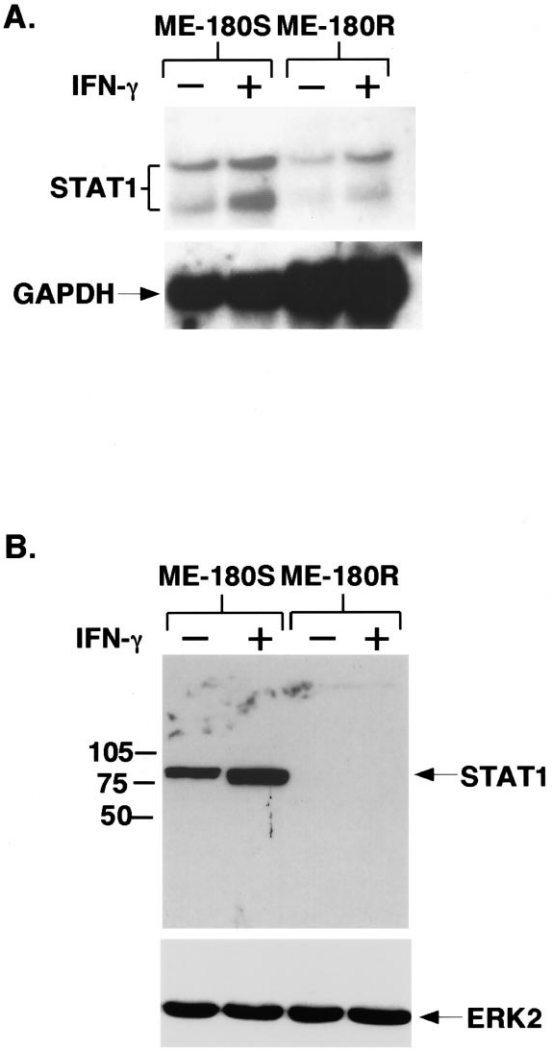

Fig. 3. STAT1 expression is markedly reduced in ME180R cells. A: Northern blot analysis of STAT1 mRNA in ME180S and ME180R cells treated with IFN-γ for 6 h. The blot was stripped and reprobed with glyceraldehyde 3-phosphate dehyrogenase (GAPDH) cDNA as a loading control. B: ME180S and ME180R cells were treated with IFN-γ also for 8 h and then harvested for Western blot analysis with a monoclonal STAT1 antibody. The blot was stripped and reprobed with an ERK2 antibody as a loading control.

(Fig. 5). There was, however, a modest induction of caspase-1 and caspase-3 in response to IFN-γ in ME180S cells which was not observed in ME180R cells (Fig. 5). These data show that STAT1 is not required for expression of constitutive caspase-1 and caspase-3 in ME180 cells but may be necessary for inducible caspase expression.

We are presently investigating the mechanism responsible for reduced STAT1 mRNA and almost complete loss of STAT1 protein in ME180R cells. We hypothesized that differences in medium contents or serum concentrations may affect the emergence of a resistant phenotype but altering culturing conditions did not prevent the emergence of resistance. It is possible that levels of regulatory proteins in the STAT1 gene which control its expression are reduced in culturing of ME180 cells. A loss of STAT1 expression has not, however, been reported in other cell lines so that ME180 cells may be unique in their response to passage in culture. There is no

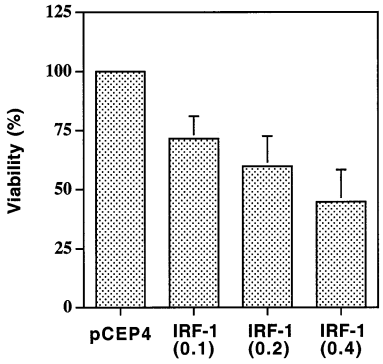

Fig. 4. Overexpression of IRF-1 induces cells death in ME180R cells. ME180R cells were transfected with increasing amounts of pCEP4-IRF-1 or pCEP4 (vector control) alone with a reporter gene (β-galactosidase). Transfected cells were stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside and examined by phase contrast microscopy 36 h after transfection. Data represent the percent viable cells which is calculated as the number of LacZ-positive cells for each condition/total number of LacZ cells in control (pCEP4). The data are the means of three experiments ± S.D.

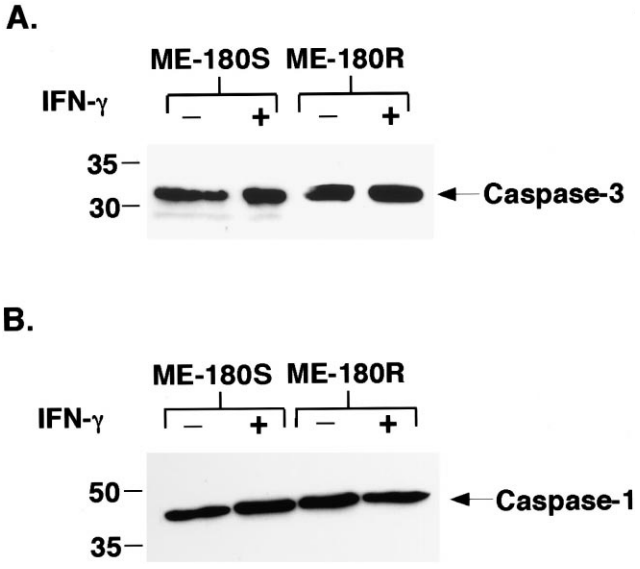

Fig. 5. Expression of basal caspase-1 and caspase-3 is similar in ME180S and ME180R cells. ME180S and ME180R cells were treated with IFN-γ for 6 h and then harvested for Western blot analysis with a monoclonal caspase-3 antibody. The blot was then stripped and reprobed with a polyclonal caspase-1 antibody.

global defect in the response of ME180R cells to apoptotic stimuli because overexpression of IRF-1 reconstitutes the cell death response.

We have shown that IFN-γ induces a caspase-mediated cell death in ME180 cells. ME180 cells become resistant to IFN-γ-induced cell death after 4–5 passages in culture. The ME180R cells are characterized by a loss of STAT1 expression and the loss of STAT1 likely results in loss of inducible IRF-1 expression. Loss of STAT1 does not, however, affect expression of constitutive levels of caspase-1 and caspase-3. It will be interesting to examine if other tumor cell lines or primary tumors show defects in STAT1 expression which may mediate resistance to cytotoxic agents.

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