IDENTIFICATION OF GRIM-19, A NOVEL CELL DEATH REGULATORY GENE INDUCED BY THE INTERFERON-BETA AND RETINOIC ACID COMBINATION, USING A GENETIC APPROACH

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RUNNING TITLE: A Novel cell death associated gene

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GenBank Accession numbers for the human and mouse GRIM-19 cDNAs described in this study are AF286697 and AF286698, respectively.
Summary:

We show here that IFN-\(\beta\)/RA combination induces death of tumor cells. To understand the molecular basis for synergistic growth-suppressive action and to identify the gene products that participate in this process, we have employed an antisense knock-out technique. This approach permits the isolation of cell death-associated genes based on their selective inactivation by overexpression of antisense cDNAs. Since the antisense mRNA inactivates gene expression of death specific genes, transfected cells survive in the presence death inducers. Several Genes associated with Retinoid-IFN induced Mortality (GRIM), were identified using this approach. Here we report the isolation of a novel GRIM gene, GRIM-19. This 552 bp long cDNA encodes a 16-kDa protein. Antisense expression of GRIM-19 confers a strong resistance against IFN/RA-induced death by reducing the intracellular levels of GRIM-19 protein. Overexpression of GRIM-19 enhances cell death in response to IFN/RA. GRIM-19 is primarily a nuclear protein, whose expression is induced by IFN/RA combination. Together, our studies identify a novel cell death regulatory molecule.

Key Words: Cell death, Cytokines, genetic approach, antisense

Abbreviations used: DAPI: 4,6, Diamidino-2-phenylindole; GRIM: Gene associated with Retinoid-Interferon induced Mortality; IFN: Interferon; IRF: IFN-gene regulatory factor; ISG: IFN-stimulated gene; ISGF: IFN-stimulated gene factor; JAK: Janus tyrosine kinase; RA: all trans retinoic acid; RAR: retinoic acid receptor; PKR: protein kinase R; STAT: Signal transducing activator of transcription; SRB: Sulforhodamine B.
INTRODUCTION:

Interferons (IFNs) represent a family of proteins that regulate antiviral, antitumor, and immune responses in the vertebrates (1,2). Type I (IFN-α and IFN-β) and type II (IFN-γ), the two major classes of IFNs, regulate these responses by inducing the expression of a number of cellular IFN-stimulated genes (ISG) upon binding to their specific high affinity cell surface receptors (3). Ligand-induced oligomerization causes the tyrosine phosphorylation of IFN receptors by protein tyrosine kinases of the JAK family. Subsequently, Signal Transducing Activators of Transcription (STAT) proteins are phosphorylated by JAKs causing the migration of the activated STATs to the nucleus and induction of gene expression. Several studies have shown that JAK1 and STAT1 are critical for type I and type II IFN-induced gene expression (3,4). Consistent with the importance of IFNs in cell growth control, disruption of STAT1 gene results in the loss of certain caspase genes of the apoptotic machinery (5). STAT1 is also critical for immune surveillance of neoplastic cells (6). Since STAT1 is a transcription factor, genes regulated by it ultimately mediate the growth suppressive actions of IFN. A number of IFN-stimulated genes (ISGs) are thought to mediate the pleiotropic effects of various IFNs (1,7). Biological functions of many of these genes remain to be clarified.

All trans retinoic acid (RA), a metabolite of vitamin-A, has profound influence on cell growth, differentiation and metabolism (8). Prolonged deprivation of vitamin A in laboratory animals results in an increased incidence of spontaneous tumors such as carcinomas. RA inhibits the growth of certain neuroblastomas, promyelocytic leukemias, and teratocarcinomas in vitro (8). Clinically, retinoids have been effective in the therapy or prevention of primary cancers of skin, head and neck (9). RA binds to specific nuclear retinoic acid receptors (RAR) and activates transcription (10). RAR acts as a transcription factor, in association with a structurally similar but genetically distinct dimerizing-partner, the retinoid X receptor (RXR), by binding to a direct repeat element, the retinoic Acid response element (RARE). Although the receptor complex is constitutively bound to RARE, several co-repressors that associate with this inhibit the gene expression complex in the unstimulated state (11). Upon engagement with ligand, the co-repressors are dislodged from this complex followed by a recruitment of several co-activators, which induce gene transcription (11). Genes controlled by this pathway are thought to mediate
the pleiotropic biological responses including growth suppression (8). In mammals, several isotypes for RARs and RXRs exist. Their expression and function are regulated in a tissue and gene specific manner (10). Several studies have shown the importance of these receptors in cell growth regulation (10,12). Like IFNs, retinoids induce the expression of a variety of genes (8,10). However, it is not clear which gene products inhibit tumor cell proliferation and apoptosis in response to retinoids.

PML-RARα, a mutant retinoic acid receptor found in certain acute promyelocytic leukemias, is generated by gene translocation (13,14). Since this receptor responds to RA, it is used as a target for the therapy of APL with RA. Interestingly, this mutant receptor is induced by IFNs and has been reported to participate in the anticellular actions of IFN-α (15-17). It forms a nuclear body consisting of several IFN-inducible gene products (18). We and others have reported earlier that in certain IFN-resistant cells, RA induces STAT1 levels leading to an enhanced IFN-response (19-21). These data suggest the existence of a cross talk between IFN- and RA-stimulated pathways, although these ligands exert their effects via disparate signaling mechanisms.

Previous studies have shown that the combination of IFN and retinoids is a potent suppressor of cell growth in vitro and in vivo, compared to either agent alone (22,23). IFN/RA-combination induces cell death in several breast carcinoma cells. To understand the molecular basis for the cell death and to identify the genes that participate in this process, we have employed a genetic technique, anti-sense technical knock-out (TKO) and identified several novel genes (24). We have named them GRIM (Genes associated with Retinoid-IFN induced Mortality), based on their function. In this report we describe the isolation and characterization of a novel gene, GRIM-19. It encodes a small, primarily nuclear protein that promotes apoptosis.

**MATERIALS & METHODS:**

**Reagents:** Restriction and DNA modifying enzymes (NE Biolabs); G418 Sulfate, IPTG (Life Technologies); Ni-NTA-sepharose (Novagen Inc); nitrocellulose membranes, ECL reagents and horseradish peroxidase coupled to anti-rabbit or anti mouse antibodies (Amersham Pharmacia Inc); goat anti-rabbit IgG-Texas red conjugate (Jackson Immunoresearch Labs Inc; human
IFN-β<sub>ser</sub> (Berlex Inc.), mouse monoclonal antibody against actin (Sigma Inc); rabbit polyclonal antibody against caspase-9 (SantaCruz Biotech Inc); Lipofectamine plus (Life Technologies Inc); Hygromycin B (Boehringer-Mannheim Inc); and multiple tissue northern blots (Clonetech Inc) were employed in these studies. Human Interferon-β was from Berlex, Inc. Fresh stocks of RA (Sigma) were prepared in ethanol and added to cultures under subdued light.

**Cell culture:** MCF-7 cells were cultured in phenol red free EMEM supplemented with 5% charcoal stripped fetal bovine serum (CSFBS) and 10<sup>-11</sup>M estradiol during treatment with IFN-β and all trans retinoic acid (RA). Cells were grown in phenol red free media 24h before treatments were initiated. All other cell lines were cultured in media with phenol red but supplemented with 5% CSFBS prior to treatment with IFN-β and RA.

**Isolation of GRIM-19 cDNA:**
Preparation of an antisense cDNA library using the RNAs derived from untreated and IFN/RA treated BT-20 breast carcinoma cell line, and selection of cells was described earlier (24). An IFN-stimulated promoter drives the expression of antisense cDNA in the episomal vector, pTKO1. Briefly, library (40 µg) was electroporated into HeLa cells (10<sup>7</sup>) and selection was initiated with Hygromycin B (200 µg/ml), human IFN-β (3000 U/ml) and RA (5µM) for 4 weeks. Parallel plates transfected with pTKO1 were selected similarly. Medium was changed and fresh drugs were added daily (first week) and then every other day. At the end of 4 weeks of selection the surviving colonies were pooled, expanded in the presence of hygromycin (200 µg/ml) and Hirt DNA extracts were prepared (25). DNA was digested with DpnI and electroporated into E. coli DH10B. The resultant colonies were screened by PCR using plasmid specific primers to detect the presence of inserts. Each individual episome was tested for cell protection against IFN/RA induced death in several breast carcinoma cell lines. Individual episomes (10µg) mixed with 30µg of salmon sperm DNA were electroporated into cells. They were then selected with IFN-β (500 U/ml), RA (1µM) and Hygromycin B (100 µg/ml) for 20 days. After that they were grown in media containing Hygromycin B alone. All cells that received pTKO1 vector died by 12 days under these conditions. Episomes that consistently conferred IFN/RA resistance were chosen for sequencing and further characterization.

**Gene expression analyses:** Northern and western blots were performed as described previously (24). In vitro transcription and translation was performed using commercially available kits (Promega Inc). GRIM-19 cDNA in pGEM-7Zf vector (Promega Inc) under the control of T7
promoter was used as a template to generate RNA. The resultant RNA was programmed into rabbit reticulocyte lysate and translated in the presence of $^{35}$S-methionine. The products were separated on a 12% SDS-polyacrylamide gel and fluorography was performed.

**Bacterial Expression and Purification of GRIM-19:** GRIM-19 ORF was amplified by polymerase chain reaction from pTKO1-GRIM-19 using the forward primer: 5’-GAAAGCTTATGCAGCGCCTAACAG-3’ and the reverse primer: 5’-GACTCGAGCTGTTACGACAT-3’. These primers contained the HindIII and XhoI sites respectively for subcloning. Purified PCR product subcloned into the bacterial expression vector pET32B (Novagen). The pET32B-GRIM-19 plasmid was transformed into E. coli BL21DE3 cells and fusion protein was induced as described earlier (24). The GRIM-19 fusion protein was purified on a Ni-NTA agarose column (Novagen) and digested with enterokinase (Invitrogen) to remove the expression tag. GRIM-19 was separated on a 12% SDS-PAGE, excised from the gel and was used to immunize rabbits. Pre- and post- immune sera were collected from the rabbits and used for western blot, immunoprecipitation and intracellular localization studies.

**Cell growth assay:** Cells (2000/well) were plated in 96-well plates. Various inhibitory agents were added and growth was monitored. Each group of treatment had 8 replicates. Cells were fixed with trichloroacetic acid (final concentration 10%) at 4° C for 1 h at the end of the experiment and stained with 0.4% sulforhodamine B (Sigma). The bound dye was eluted with 100µl of Tris-HCl (pH 10.5) and the absorbance was monitored at 570 nm. One plate was fixed with TCA, 6 h after plating the cells. Absorbance obtained with this plate is taken as 0% growth. Absorbance obtained with wells containing untreated cells was taken as 100% growth. An increase and decrease of A570 values in the experimental wells relative to the initial value will indicate cell growth and death, respectively. When plotted as percentage of untreated control growth and death values appear on the positive and negative scales of the Y-axis, respectively. Annexin-V binding assays were performed using a commercially available kit (Trevigen Inc) per manufacturer’s recommendation.

**Transient transfection assay:** HeLa cells were transfected with the indicated plasmids (1µg) using the Lipofectamine plus reagent (Life Technologies Inc) according to manufacturer’s recommendations in chamber slides. Cells were treated with various agents for 72 h, fixed and stained with DAPI (0.2µg/ml). Percentage of cells with fragmented nuclei was determined from multiple fields.
Caspase assay: To determine the enzymatic activity of caspase-9, a commercially available colorimetric assay kit (Biosource International) was employed. Briefly, cell lysates were prepared after various treatments and a comparable quantity of lysate from each sample was incubated with the synthetic substrate LEHD-pNA. The release of chromophore, pNA (p-nitroanilide), from the substrate was quantified by monitoring the absorbance at 405 nm in a microplate reader. Assay was performed as recommended by the manufacturer.

Over expression of GRIM-19 in mammalian cells:
GRIM-19 and its mutant were cloned into pCXN2-myc vector by PCR using primers specific sequence GRIM-19. Restriction sites were added at the 5’ and 3’ to permit their cloning. The 5’ (forward) primer: 5’-GCGGAATTCCACCATGGCGGCGTCAAGGG-3’ contains an EcoRI site followed by a Kozak consensus site. The 3’ (reverse) primer sequence 5’-GCAGGCTACCGTACCATGAAGGCCG-3’, contains a KpnI site. The primer used to make the 3’ deletion is as follows: GRIM-19Δ1 was generated by using a forward primer, described above, and the following reverse primer: 5’-GCAGGCTACCGTACCATGATGATGCGCTCTC-3’. Following PCR, the products were digested with EcoRI and KpnI and subcloned into the mammalian expression vector pCXN2-myc, in which a C-terminal myc-tag is added to the protein (D.J. Lindner and D.V. Kalvakolanu unpublished data). This vector also carries a neomycin selection marker for generating stable cell lines.

Immunoprecipitation:
Cells at 70% confluence in EMEM 5% NCS were treated with human IFN-β (500 U/ml) and RA (1µM) for the indicated times. Plates were then washed extensively with PBS. They were grown in EMEM without methionine and cysteine for additional 2h. 35S labeled methionine and cysteine (Amersham “ProMix” cat #SJQ0079) was added (50uCi/ml) and incubated at 37 °C for 3h. Cells were lysed in ice-cold RIPA buffer containing protease inhibitors (2.5ug/ml Leupeptin, 10ug/ml Aprotinin, and 0.5M PMSF). Cell extract (200µg) from each sample was immunoprecipitated using rabbit polyclonal IgG specific for GRIM-19 (1:100 dilution) at 4°C overnight with gentle rocking. In each case a parallel sample was immunoprecipitated using preimmune IgG from the same rabbit. Protein-G Sepharose (200ul of 1mg/ml stock, Sigma) was added to the immune complexes and incubated with rocking at 4 °C for 3hrs. The bound complexes were centrifuged and the beads were washed 1 time with RIPA buffer then 2 times with wash buffer (0.5M NaCl, 10mM Tris pH 8.0, 0.1% NP40). The final pellet was suspended
in loading buffer (30μl) and boiled for 5min before separating on a 12% SDS-polyacrylamide gel. Gels were fixed in 30% Methanol, 10% Glacial Acetic Acid, and 15% Glycerol for 30 minutes, followed by soaking in a commercially available fluorographic reagent, Amplify (Amersham), dried and fluorographed. Radioactivity in the bands was quantified using a Molecular Dynamics phosphorimager.

**Immunofluorescence:**
Cells grown in chamber slides (Nalgene) were fixed with 2% paraformaldehyde in PBS for 10 min and then with methanol for 20min at room temperature (26). The cells were then washed thrice with PBS containing 0.15% glycine, and 0.5% BSA. The cells were then incubated with antibodies specific for GRIM-19 (1:250 dilution) in the same buffer for 1 hour at 37°C. Cells were washed with PBS and then incubated with Texas red-labeled goat anti-rabbit IgG (1:200 dilution) for 1h. They were then washed with PBS and observed under a fluorescent microscope. These cells were also counter stained with DAPI (0.2μg/ml) to visualize the nuclei. In certain experiments the primary antibody was pre-incubated with bacterially expressed GRIM-19 (5μg) for 1hr before using in the experiment.

**RESULTS:**

**Induction of cell death by the IFN/RA combination in breast carcinoma cells:**
IFNs have been shown to be less effective in the therapy of solid tumor cell growth, compared to hematologic tumors such as chronic myelogenous leukemia (27,28). To determine whether RA would enhance the growth suppression, we treated MCF-7 cells with IFN−β, RA, or IFN−β/RA for various days and measured cell death using the Sulforhodamine B (SRB) binding assay (29). As shown in Figure1A, either IFN−β (250U/ml) or RA (1μM) alone marginally suppressed the growth, but did not induce death of MCF-7 cells. In contrast, the combination of IFN/RA caused a time-dependent increase in cell death. By 10-12 days all the cells in the culture were killed by IFN/RA combination. Annexin-V binding assays were conducted to determine whether the cell death was similar to apoptosis (Figure 1B). Exposure of cells to IFN or RA alone did not cause significant death, compared to the untreated ones. However, the IFN/RA combination caused a dramatic increase in annexin-V positive cells. Prolonged exposure of cells to the combination
increased the annexin-V positive cells (Figure 1C, compare bars 2, 3, and 4 to bar 1), consistent with the data in panel A.

A genetic approach to identify the Genes associated with Retinoid-IFN induced Mortality (GRIM): Our previous studies have not indicated any evidence for the activation of certain IFN regulated gene products such as the Ribonuclease L (RNAseL) and protein kinase R (PKR) during IFN/RA-induced death (24). Similarly, we did not observe any changes in the levels of certain members of the BCL family of death regulators and of tumor suppressor p53 (data not shown). Therefore, we hypothesized that hitherto undefined gene products are activated during IFN/RA induced cell death. To identify the GRIMs we have employed a genetic approach. In this approach, overexpression of an antisense cDNA library by an episomal vector inactivates specific death-regulatory genes (30). Consequently, only those cells that have been transfected with the death-associated genes (expressed in an antisense orientation) will survive and continue to grow in the presence of death activating agents. This provides a powerful forward selection to rescue the genes. Genes rescued from the first round of selection are then transfected into cells individually to confirm their ability to confer resistance to ligand induced death.

An antisense cDNA library of BT-20 cells was generated in the episomal vector, pTKO1 (24). Electroporation of this library (containing ~5X10^6 cDNA inserts of various size) into HeLa cells, followed by selection of the cells with IFN-β (3000U/ml), RA (5µM) and Hygromycin-B (200µg/ml) for 4 weeks yielded several drug resistant colonies (24). Under the same conditions, cells transfected with the episomal vector alone did not yield any surviving colonies. The episomes were rescued from the pooled colonies by the Hirt extraction followed by transformation into E.Coli DH10B. Single episomes rescued from this round were further tested for their ability to confer similar resistance to IFN (500U/ml)/RA (1µM) and Hygromycin-B (100 µg/ml) into several breast carcinoma cells including MCF-7, and T47D. Those that conferred resistance to IFN/RA were chosen for characterization. We have selected the smallest of these cDNAs, GRIM-19, for this study. The name stands for the function of the gene and the number indicates the original number assigned to the episome. Transfection of GRIM-19
episome into MCF-7 cells and subsequent selection with IFN–β/RA and Hygromycin-B for 3 weeks resulted in the survival of a number of colonies (Figure 2A). Under these conditions none of the cells transfected with the episomal vector, pTKO1, survived IFN-RA treatment. Similar results were obtained with T47D and BT-20 cells. Thus, GRIM-19 appears to be a death-associated gene.

To further prove the growth promoting effects of antisense GRIM-19, two HeLa cell lines, which expressed the pTKO1 vector alone or the GRIM-19 in an antisense orientation were prepared. These cells were exposed to the combination of IFN-β (1500U/ml) and retinoic acid (2μM) for several days and the growth was monitored using the SRB assay. Antisense GRIM-19 was capable of conferring a strong growth advantage to cells as detected by an increase in the absorbance (Figure 2B). The vector-transfected cells failed to grow under these conditions. Prolonged exposure of these cells to IFN/RA caused death of vector-transfected cells. Similar data were obtained in other tumor cells (data not shown).

**Characterization of GRIM-19 gene:** The cDNA in the episome was sequenced using vector-specific primers. A 450 bp long insert was obtained from the GRIM-19 episome. Since it appeared to be smaller than the mRNA predicted in northern blots (see below), it was used as probe to screen a human BT-20 cell library carrying full-length cDNAs in normal orientation. The resultant cDNA was 552 bp long. It has an open reading frame capable of coding for a protein of 144 aminoacids (Figure 3). The cDNA has a 7 and 93 base pair long 5’ and 3’ UTRs, respectively. The first ATG is in the context of a Kozak translational initiation site. A consensus polyadenylation signal, AAUAAA is present at position 506. We also have isolated a mouse homologue of GRIM-19 (Figure 3). GenBank searches identified several uncharacterized sequences, which possessed homology to GRIM-19. Among these are a cDNA from *Arabidopsis thaliana* (GenBank accession 049313 and 023022) and another from *Caenorhabditis elegans* (GB accession 044955) and two other human sequences (GenBank accession numbers AF132973 and AF155662). A distantly related protein is also present in the bacterium *Staphylococcus aureus*. Most of these proteins have 138–204 aminoacids, with the Arabidopsis homologue being the longest. The *C. elegans* and *A. thaliana* homologues of GRIM-19 exhibited 32% identity and 50% similarity with human GRIM-19 at the aminoacid level. The mouse
GRIM-19 is 75% identical and 88% similar to human protein. Thus, GRIM-19-like genes appear to be present in most eukaryotes. The human and mouse cDNAs sizes match with their corresponding mRNAs. EST database searches did not yield any other related sequences. Northern blot analyses identified a single mRNA species (see below).

**Expression and generation of antibodies specific to GRIM-19 protein:**

The predicted protein has a $M_r$ 16.0 kDa and a pI of 8.02. To experimentally determine the size of the protein encoded by the GRIM-19 cDNA, we have subcloned the insert into pGEM-7Zf under the control of T7 promoter. RNA generated from this construct was translated in vitro using rabbit reticulocyte lysates. SDS-PAGE analysis of the translation products revealed the presence of a ~16 kDa protein in the lanes programmed with RNA of GRIM-19, but not in those with vector derived RNA (Figure 4A). To gain a better understanding into the antisense mediated inhibition of GRIM-19, we raised antibodies against the GRIM-19 protein. The open reading frame corresponding to the GRIM-19 was subcloned into bacterial expression vector pET32B. The recombinant was expressed as a His-TAG fusion protein and purified. The protein was analyzed by SDS-PAGE followed by silver staining analysis (Figure 4B). The purified protein, after removal of expression tag, was used for raising antibodies in rabbits. These antibodies were used for western blot analysis. As shown in Figure 4D, these antibodies specifically detected GRIM-19 but not the Tag protein. Pre-immune sera did not detect any protein (Figure 4C).

**Inhibition of GRIM-19 gene expression by antisense cDNA:**

To provide a direct evidence for the repression of GRIM-19 protein by antisense, northern blot analyses were performed. These assays were performed using the RNA derived from HeLa and MCF-7 cells. For conducting these experiments, a control cell line was generated by transfecting the pTKO1 vector and selecting the cells with Hygromycin-B. Stable cell lines were treated for 3 days with IFN-β (500U/ml) and RA (1 µM), RNA was extracted, and northern blots were prepared. These blots were probed with a $^{32}$P-labeled GRIM-19 cDNA. As anticipated the vector transfected HeLa cells expressed a fast moving RNA of about 550bp (marked as E in Figure 5A). In contrast, a fast and a slow moving band were detected in cells transfected with GRIM-19 cDNA (Figure 5A). These correspond to the sense and antisense mRNAs resulting
from the endogenous and transfected genes, respectively. Similar data was obtained from MCF-7 cells (Figure 5C).

We next determined whether antisense expression caused a reduction in the levels of endogenous GRIM-19 protein. Cell extracts were prepared and equal amounts of cell extracts were used in western blots. These blots were probed with antibodies described in Figure 4D. While a normal expression of the GRIM-19 protein was seen in the vector-transfected cells, it was completely repressed in cells transfected with the antisense-GRIM-19 episome (Figure 5B and 5D). A parallel blot containing the same extracts was probed with a monoclonal antibody raised against IFN-regulated RNaseL. The levels of RNaseL, however, were unchanged in both the cases (Figure 5E).

**Induction of GRIM-19 expression by IFN/RA combination:** Since GRIM-19 was isolated as a gene involved in IFN/RA induced death pathway, we examined the influence of IFN/RA on GRIM-19 gene expression in several breast carcinoma cell lines. Cells were treated with IFN-β (500U/ml) + RA (1µM) for various lengths of time, RNA was extracted, blotted and probed with a labeled human GRIM-19 cDNA. As shown in Figure 6, a single species of GRIM-19 mRNA (~550 bp) was inducible in a variety of breast carcinoma cells, irrespective of their estrogen responsiveness (see panels A, B, C). However, unlike the known IFN-stimulated genes, the induction of GRIM-19 is slower with a significant induction occurring at 24h (~5 fold) and reaching the maximum at 48-72h post IFN/RA treatment.

Although the combination of two ligands induced GRIM-19, it was unclear whether IFN or RA alone capable of inducing the gene expression. Therefore, MCF-7 cells were treated with either IFN-β or RA or the combination. RNA was extracted, blotted and probed with human GRIM-19 cDNA. Expression of GRIM-19 was induced 3-fold by IFN-β. RA alone did not induce the expression. However, the combination of IFN/RA robustly (8-fold) induced the GRIM-19 mRNA expression (Figure 6D).

We next examined whether an increase in GRIM-19 mRNA level also corresponded to a similar increase in its protein levels. MCF-7 cells were labeled with $^{35}$S-methionine in the
presence of IFN/RA combination for various lengths of time. Equal amounts of Labeled cell extracts were immunoprecipitated with pre-immune and post-immune IgG (Figure 6E). Pre-immune IgG did not precipitate any proteins in the 16-kDa range. However, post-immune IgG precipitated a protein of ~16kDa, whose level increased as a function of IFN/RA-treatment. Accumulation of GRIM-19 protein paralleled the mRNA induction kinetics (Figure 6A) with approximately 12-fold increase in protein levels occurring at 72h. Interestingly, several other proteins also immunoprecipitated along with the GRIM-19 protein in the lysate (Figure 6E, last lane). These proteins were not precipitated by pre-immune IgG. Interaction of other proteins with GRIM-19 was also confirmed by immunoprecipitation of cells transfected with a myc-tagged GRIM-19 (data not shown).

The specificity of the antibody in the recognition of GRIM-19 protein was tested in a competitive immunoprecipitation assay (Figure 6F). Comparable amounts of $^{35}$S-labeled, IFN/RA treated (48h) were incubated with pre-immune or post-immune IgG and immunoprecipitation was carried out. As anticipated, only the post-immune IgG selectively immunoprecipitated the labeled GRIM-19 protein (compare lanes 1 and 3 of figure 6F). In a parallel experiment, preincubation of the post-immune IgG (lane 4) with a bacterially expressed, purified recombinant GRIM-19 (rGRIM-19) inhibited the immunoprecipitation of labeled cellular GRIM-19. As anticipated rGRIM-19 had no effect on pre-immune IgG (lane 2).

We next determined the induction of GRIM-19 protein by individual agents. MCF-7 cells were treated with IFN-β, RA or their combination (as in figure 6D), equal amount of protein from each sample was western blotted with GRIM-19 specific antibodies. Consistent with the northern data, western blot analyses IFN-β induced the GRIM-19 protein slightly (2.5 fold). The IFN/RA combination caused a maximal (~7 fold) in this experiment (Figure 6G). RA alone did not induce the protein significantly compared to the untreated cells. All lanes had comparable amount of protein as determined by western analysis with antibodies specific to actin.

**Tissue distribution of GRIM-19 mRNA:**

To provide further evidence that the GRIM-19 cDNA was not a cloning artifact, we probed commercially available northern blots containing poly A$^+$ RNA from multiple human tissues.
(Clonetech Inc). Although the GRIM-19 is detectable in a wide variety of human tissues, a tissue-specific variation is observed (Figure 3A). Notably, the human heart, skeletal muscle, liver and kidney and placenta expressed higher steady state levels of GRIM-19, relative to other tissues. Human Lung, peripheral blood leukocytes, spleen and thymus and colon had the lowest GRIM-19 expression. These blots were also probed with β-actin to ensure the presence of comparable amount of RNA in all the tracks (Figure 7B). Consistent with the reports of the presence of two different forms of actin in human cardiac and skeletal muscles, these can be readily detected in those tissues but not in others. In the cells of human hematopoietic system, GRIM-19 is present in variable amounts. In fetal liver, spleen, and bone marrow highest level of GRIM-19 was observed (Figure 7C).

**Overexpression of GRIM-19 enhances cell death:**

Since IFN/RA treatment induced GRIM-19 mRNA and protein expression and downregulation of GRIM-19 provided growth advantage to the cells, it was of interest to determine the effects of over expression of GRIM-19 in cells. The GRIM-19 cDNA was subcloned into pCXN2 vector in which the transgene expression was regulated by the actin gene enhancer (31). This vector was modified to add a C-terminal myc-tag to the proteins expressed from cloned inserts (Lindner and Kalvakolanu, unpublished). In the first set of experiments, cells were transfected with a comparable molar quantity of either vector alone or the vector expressing GRIM-19. Total number of G418 resistant colonies formed at the end of 3 weeks of selection was determined. Significantly fewer colonies (26% less) formed upon overexpression of GRIM-19 compared to the vector control (Figure 8A).

Surviving colonies were pooled and analyzed by northern and western blotting for the expression of mRNA and protein, respectively. As shown in figures 8B and 8C, these cells expressed detectable levels of GRIM-19 mRNA and its protein. Northern blot analysis detected a new mRNA, in addition to the endogenous one, in the GRIM-19 transfected cells only. Western blotting with anti-myc tag antibodies detected an expected sized protein in the GRIM-19 transfected cells but not in the vector transfected ones. However, a high quantity of the cellular protein (~300 µg) was required to detect the protein. Since the antibodies are directed against the myc-tag, only the transgene-derived GRIM-19 protein but not the one from endogenous gene, is
detected in these blots. We next examined the effects of exogenous GRIM-19 expression on cell growth in the presence of IFN-β/RA combination (Figure 8D). Vector and GRIM-19 expressing cells were treated with IFN/RA combination for 96h and then stained with FITC tagged annexin-V. Annexin-V positive cells were scored by FACS analysis. Cells transfected with GRIM-19 were hypersensitive (~3.5 fold) to the death-inductive effects of IFN/RA combination. RA did not significantly affect the growth of cells overexpressing GRIM-19, compared to the vector transfected ones (Figure 8E). Interestingly, IFN-β potently suppressed the growth of cells overexpressing GRIM-19 but not vector transfected ones. To confirm the apoptotic nature of GRIM-19 dependent cell death, we tested the effect of a cell permeable caspase inhibitor, z-VAD-fmk, on cell death (Figure 8F). GRIM-19 overexpressing cells were pretreated with IFN/RA combination in the absence or presence of two different concentrations of the inhibitor for 96h. While a high amount of cell death can be seen in the absence of the caspase inhibitor, it was suppressed in a dose-dependent manner.

One hallmark of cell death is the activation of caspases (32,33). These enzymes cleave various cellular proteins, thus causing a loss of cell viability. The apoptotic nature of GRIM-19 augmented cell death was also confirmed by measuring the caspase-9 activity in MCF-7 cells expressing the vector or GRIM-19. Caspase-9 was chosen for this purpose, since a number of studies have shown the importance of this enzyme for multiple death pathways (32,33). After treatment with various drugs, cell lysates were prepared and caspase-9 activity was measured by colorimetric assay. In this assay, the cleavage of a synthetic substrate (34), LEHD-pNA, by caspase-9 releases the chromophore pNA (p-nitroanilide). Absorbance of the chromophore at 405 nm was monitored in a plate reader. The data is shown in figure 8G. In response to IFN/RA-treatment caspase-9 activity was higher in cells expressing GRIM-19, compared to the vector transfected cells. A maximal caspase-9 activity (6 fold) was noted in IFN/RA-treated vector expressing cells, compared to those that received no treatment, IFN or RA. IFN did not significantly affect caspase-9 activity in these cells. In contrast, IFN-β treatment increased ~3 fold in GRIM-19 expressing cells. RA did not cause any change in Caspase-9 activity. In the IFN/RA-treated GRIM-19 expressing cells, caspase-9 activity was increased 15-fold, compared to the untreated ones. This was significantly higher than that observed in the vector-transfected cells treated similarly. No changes in Caspase-9 protein levels were noted in western blots.
There was no detectable cleavage of caspase-9 under these conditions. This observation is not unusual, since recent reports have shown that caspase-9 can be activated without being proteolytically processed (35).

Since MCF-7 cells lack caspase-3 (36), a critical mediator of DNA fragmentation, we were unable to demonstrate the internucleosomal fragmentation of DNA. Because HeLa cells have a normal caspase-3, we employed a transient transfection assay, in which DNA fragmentation was monitored microscopically after DAPI staining. Cells were transfected with the vector or GRIM-19 cDNA. A representative DAPI stained field is shown in figure 9A. In the parallel experiment, cells were treated with IFN/RA combination for 72h and the percentage of cells with fragmented nuclei was determined. (Figure 9B) Although transfection of vector alone caused a basal level of DNA fragmentation, treatment with IFN/RA increased the number of cells fragmented nuclei. Transfection of GRIM-19 expression vector significantly elevated cells with the nuclear fragmentation, compared to the vector control. GRIM-19 transfected cells were hypersensitive to death as revealed by a strong increment in the percentage of cells with fragmented nuclei. These data indicate that GRIM-19 undergoes post-translational modifications and/or interacts with other factors in the presence of IFN/RA. Such activities contribute to the overall magnitude of cell death.

The effect of individual agents, IFN-β and RA, was also determined in a similar assay (Figure 9C). Cells after transfection with the GRIM-19 expression vector were stained with DAPI and the number of cells with fragmented nuclei was determined. Cell death observed in untreated control (11%) was subtracted from the experimental samples. IFN-β caused a slight but significant increase in cells with fragmented nuclei in GRIM-19 transfected cells. RA alone did not enhance cell death. However, the IFN/RA combination caused a dramatic increase in the number of cells with fragmented nuclei. These data are consistent with observations that IFN/RA combination is a potent inducer of cell death compared to either drug alone. The differences in the percentage of dead cells between Figures 9A and 9B may be due to variance in transfection efficiency between different experiments.
Deletion of the C-terminus of GRIM-19 inhibits its death promoting function:

To define the functional domain(s) of GRIM-19 important for its death regulatory actions, a mutant lacking the C-terminus (GRIM-19Δ1) was generated. This mutant was arbitrarily designed, by removing the C-terminal 43 amino acids and was expressed as a myc-epitope tagged fusion protein in MCF-7 cells (Figure 10A). Effect of the mutant on IFN/RA-induced death was determined by comparing the relative cell death occurred in the vector, GRIM-19, and GRIM-19Δ1 transfected cells (Figure 10B). Although IFN/RA caused death in all the cells, a greater amount of cell death occurred in the GRIM-19 transfected cells compared to vector-transfected cells. However, a similar magnitude of death was noted in GRIM-19Δ1 and vector transfected cells upon, IFN/RA-treatment, even though it expressed 5-6 fold more than the wild type protein. Additional truncation of the ORF did not further reveal any other death promoting domains (data not shown). Deletion of N-terminal 50 amino acids also did not inhibit death (N.V. Chidambaram et al manuscript in preparation) Thus, the C-terminus of GRIM-19 appears to be important for inducing death.

Intracellular localization of GRIM-19: The intracellular location of GRIM-19 was determined by immunofluorescence (26) experiments. HeLa cells were probed with GRIM-19 specific IgG. As shown in figure 11A, GRIM-19 specific antibodies intensely stained the nucleus, although some punctate cytoplasmic staining could also be seen. To ascertain the specificity of such detection, the post-immune IgG was pre-incubated with recombinant GRIM-19 protein. The IgG was then used for staining the cells (Figure 11B). The recombinant protein completely blocked the staining. These cells were also counter stained with DAPI to visualize the nucleus. Thus, GRIM-19 appears to be primarily a nuclear protein. Similar results were obtained with other cell types.

We next examined whether IFN/RA caused a change in intracellular localization of GRIM-19 (Figure 12). HeLa cells were treated with IFN-β (3000U/ml), RA (5µM) or the combination for 4 days. They were fixed and immunostained with antiGRIM-19 antibody and DAPI. No significant change in the intracellular localization of GRIM-19 was detected following the exposure of cells to individual drugs, compared to the vector. Consistent with the high
induction of GRIM-19 gene, cells treated with IFN/RA combination were stained strongly by the antibody. The IFN/RA caused the fragmentation of nucleus. However, GRIM-19 protein was still in the nucleoplasm. Interestingly the GRIM-19 was excluded from the condensed nuclear fragments.

**DISCUSSION:**

Given the multitude of ISGs and RA-responsive genes it has been difficult to precisely pinpoint the gene products that mediate the growth suppressive or cell death regulatory functions of Interferons or retinoids. Therefore, application of genetic techniques would be useful in identifying the critical regulators of IFN/RA-induced death. In this study, we have shown that IFN/RA combination is cytotoxic to several tumor cell lines (Figure 1 and data not shown). Although a generalized loss of nuclear integrity was seen, internucleosomal fragmentation, a classic feature of apoptosis, was not observed in MCF-7 cells. Indeed, in several forms of apoptosis DNA fragmentation is dispensable (37-40). Furthermore MCF-7 have a 47-nt deletion in Caspase 3 gene, a critical protease necessary for promoting DNA fragmentation (36). IFN/RA combination kills these cells. Recent studies also show that proteases other than the caspases mediate cell death in response to IFN-\(\gamma\) (41). Moreover, two of the previously suspected IFN-stimulated growth regulators, RNAseL and PKR, did not appear to have a role in IFN/RA-induced death (24). Similarly, no elevation of p53 and BCL proteins occurred under the conditions of cell death (data not presented). These observations suggest the existence of undefined mechanisms of cell death. Therefore, we have hypothesized that novel Gene(s) associated with Retinoid-IFN induced Mortality (GRIM) mediate the cytotoxic effect. Since two different biological response modifiers are required to activate cell death, it is likely that several steps are involved in the upregulation of GRIMs.

To identify the GRIMs, we have employed an antisense knock-out method, which permits the isolation of death-associated genes by a functional inactivation (30). Our preliminary sequence analyses suggest that GRIMs do not include any of the known death-activating genes (24). This observation suggests several possibilities. First, the previously described death activators do not regulate IFN/RA induced apoptosis. Second, they cannot be inactivated by
antisense approaches. Third, such gene products are at the terminal phase of death pathway and their inactivation is insufficient to overcome the death. The rescue of multiple GRIMs in our studies suggests that the IFN/RA-combination uses a death pathway consisting of several steps. It is likely these GRIMs constitute a single linear pathway with multiple branch points or multiple parallel pathways that converge at various points. Consequently inactivation of a single GRIM is sufficient to suppress cell death. One of the GRIMs is a redox-regulatory enzyme, thioredoxin reductase (GRIM-12) (24). In this study we described GRIM-19. Complete characterization of other GRIMs would clearly aid in establishing the various steps involved in the death pathway. Our approach seeks to identify GRIMs irrespective of their inducibility, in an unbiased manner. The antisense library included cDNAs derived from pre- and post- IFN/RA treatment of cells. Thus, a GRIM gene need not be induced at a transcriptional level. This pattern of control is exemplified by one of the GRIMs. While the mRNA coding for GRIM-19 is inducible (Figure 6) that of GRIM-12 is not (24). Since an IFN-Stimulated Response Element (ISRE) drives the expression of antisense cDNAs, a functional JAK-STAT pathway is necessary for conferring resistance. Therefore, only the genes downstream of the JAK-STAT pathway will be inactivated by antisense expression.

GRIM-19 is a novel death regulatory gene, whose inactivation confers growth advantage to cells in the presence of IFN/RA (figure 2). Since EST database searches to date have not revealed additional homologues, there appears to be a single gene for GRIM-19. Northern blot analysis also showed a single mRNA for GRIM-19. Chromosomal mapping studies revealed a single locus on human chromosome 19 (N.V. Chidambaram et al J. Interferon & Cytokine Research in press). Thus, the growth promoting effect of antisense GRIM-19 is primarily due to the inactivation of a single GRIM-19 gene. Consistent with its death regulatory functions, overexpression of GRIM-19 reduces cell viability as seen by the formation of fewer G418-resistant colonies (figure 8A). Cells that express moderate levels of GRIM-19 are significantly more susceptible to the cytotoxic action of IFN/RA. A truncated GRIM-19 fails to augment cell death (Figure 10B). Further studies are in progress to analyze the function of this domain. Apoptotic nature of GRIM-19 dependent cell death was confirmed by the annexin-V staining of dying cells and its inhibition by caspase inhibitor, z-VAD-fmk (figure 8F). Hyperactivation of caspase-9 enzymatic activity in IFN/RA treated GRIM-19-transfected cells is consistent with the
apoptotic nature of GRIM-19 protein (figure 8G). The increase in caspase-9 activity does not appear to be due to an increase in its level under the conditions of treatment (Figure 8H).

Similarly, induction of DNA fragmentation in HeLa cells by transfected GRIM-19 (figure 9) supports the notion that GRIM-19 induces apoptosis under the control of IFN/RA combination.

Growth promotion by IFN/RA combination in the antisense GRIM-19 expressing cells is not a cell type or clone specific effect. The experiments in figures 2 and 5 were conducted with pools of clones derived from HeLa or MCF-7 cells. Inactivation of GRIM-19 alone causes growth promotion (Figures 2 and 5). The levels of RNAseL, an IFN induced growth suppressive ribonuclease (42), are unaffected by antisense GRIM-19 expression (Figure 5E). GRIM-19 maps to human chromosome 19 p13.1 (N.V. Chidambram et al J. Interferon & Cytokine Research in press) a region essential for prostate tumor suppression (43) and thus may represent a novel tumor suppressor. Lastly, the presence of GRIM-19 homologues in other eukaryotes suggests the existence of a similar, if not identical, death pathway in these organisms. It will be interesting to determine what physiologic cues activate these molecules in the respective organisms.

The presence of GRIM-19 mRNA in normal tissues suggests that it is not a cloning artifact. Surprisingly, some tissues such as heart, skeletal muscle and kidney express a high steady state level of GRIM-19 mRNA. Such expression may not mean that these tissues undergo apoptosis spontaneously. It is important to emphasize that GRIM-19 interacts with other proteins and undergoes phosphorylation (figure 6E and data not shown). This would suggest that its activity is regulated by other factors. Therefore, in the absence of exogenous cues it may not activate death. Indeed, the IFN/RA combination requires multiple gene products to execute cell death as suggested by the rescue of multiple GRIMs (24). Thus, a concerted action of these products is critical for mediating cell death. Therefore, higher expression of a single GRIM is insufficient to induce death. However, consistent with its role in death pathways, overexpression of GRIM-19 enhances the cellular sensitivity to IFN/RA induced death (Figures 8 and 9). That said, GRIM-19 might participate in other unknown physiological functions in the absence of death cues.
IFN/RA-combination upregulates GRIM-19 levels during cell death in a variety of tumor cells (Figure 6A, B, C). The fact that GRIM-19 is not inducible by RA (Figure 6D) suggests that RA is not a direct regulator of GRIM-19. RA may inhibit the function of a negative effector of IFN-induced response, thereby augmenting GRIM-19 gene expression. This notion is also supported by two observations. 1) IFN-β alone induces GRIM-19 gene expression by ~2.5 fold (Figure 6D) and IFN-β suppresses cell growth in cells that over express GRIM-19 (Figure 8B). 2) Although RA does not induce GRIM-19 gene, it enhances IFN-induced expression. RA alone does not significantly enhance cell growth suppression in GRIM-19 expressing cells (Figure 8). The mechanism of GRIM-19 gene regulation by IFN/RA is currently being investigated.

Deletion studies have identified that the C-terminus of GRIM-19 is essential for cell death induction. This region harbors a putative ATP binding domain, which is well conserved in all the eukaryotic GRIM-19 like gene products (Figure 3). The deletion terminates the cDNA in the center of the conserved ATP binding domain, IMKDVPXWKG. It is likely ATP binding is necessary for exerting the death functions. Preliminary studies indicate that ATP binds to GRIM-19 protein (data not shown). Studies are in progress to define the function of this domain. Interestingly, truncated protein can be expressed in higher amounts than the wild type protein (Figure 9A) in several experiments. This difference could be due to a lesser cytotoxicity of the mutant protein, compared to the wild type.

Nucleotide binding proteins, in particular to those that bind to GTP seem to play specific roles in the IFN actions. Some of these, such as the Mx (44) and TGTP (45) act as specific antiviral proteins against myxoviral and rhabdoviruses, respectively. Others such as IGTP are required for anti protozoan action (46). Functions of other IFN inducible GTP binding proteins such as LRG-47 (47), IRG-47 (48), and IIGP (49) are unknown. However, these proteins are of 47-kDa and appear to involve in vesicle transport or located in the endoplasmic reticulum of the cells (44,46). Contrary to these, GRIM-19 is a small, predominantly nuclear protein (Figure 11). We speculate that it may aid in functions such as importing of death activating proteins into the nucleus. Its activity may be regulated protein kinases in response to exogenous death cues. Preliminary studies indicate that GRIM-19 protein is constitutively phosphorylated and IFN/RA combination hyperinduces it (data not presented). A small amount of this protein is also in the
cytoplasm. Therefore, it may mediate other death related functions in the cytoplasm.

Immunofluorescence analysis with the available polyclonal antibodies revealed no obvious differences in the intracellular distribution of GRIM-19 protein following IFN/RA treatment. The specific role GRIM-19 plays in the nucleus remains to be investigated further.

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References:


**Legends to Figures:**

**Figure 1:** Cell death induction by the IFN-β and retinoic acid combination. (A) Cell growth was measured using sulforhodamine B as described in Materials and Methods (29). Absorbance (570 nm) of bound dye was quantified and growth percent was calculated. Each data point represents mean ± SE of six replicates. The absorbance value for 0% growth is 0.158. The 100% growth values for days 2, 4, 6, 8 and 10 are 0.279; 0.397; 0.712; 1.278 and 1.836, respectively. Empty bars: No treatment; Dotted bars: RA (1µM); Striped bars IFN-β (500U/ml); Black bars: IFN-β/RA combination. Values on negative scale indicate death of initially plated cells. (B) IFN/RA induced apoptosis. MCF-7 cells were exposed to various treatments for 1 day and then stained with propidium iodide and FITC-labeled annexin-V. Double-positive cells were scored by FACS analysis and calculated as the percent of total propidium iodide-positive cells. Bar notations are similar to panel A. IFN-β and RA concentrations are similar to those in panel A. In panel C cells were treated with IFN-β and RA for the indicated days and FACS analysis was performed as described earlier (50).

**Figure 2:** MCF-7 cells transfected with antisense GRIM-19 become resistant to death induced by IFN/RA (A). Cells were electroporated with 10 µg of expression vector pTKO1 or the same vector carrying GRIM-19 cDNA in an antisense orientation. Cells were selected with hygromycin B (75µg/ml), IFN-β (500U/ml) and RA (1µM) for 3 weeks and then with hygromycin B alone for 1 week. Colonies surviving on the plates were fixed and stained with Sulforhodamine B as described above. Expression of antisense GRIM-19 in cells confers growth
advantage to the cells. (B) HeLa cells were stably transfected with pTKO1 (triangles) or pTKO1-GRIM-19 (squares) and selected with hygromycin B. The surviving colonies (~300 each) were pooled separately. Each pool of cells was plated in 96 wells and their growth was monitored in the presence of IFN-β (1500U/ml) and RA (2µM) for the days indicated on X-axis. Growth assay was performed as in Figure 1. An increase in absorbance indicates the cell growth. Each data point represents mean ± SE of five replicates.

**Figure 3:** Sequences of the human and murine GRIM-19 proteins. ClustalW alignments of the GRIM-19 like proteins from various sources. Note the conservation of sequences between eukaryotic GRIM-19 sequences. An assignment of the name “GRIM-19” to non-mammalian homologues is solely based on sequence homologies, not on function.

**Figure 4:** Expression and preparation of antibodies specific to GRIM-19. (A) Human GRIM-19 cDNA was cloned under the control of T7 promoter in pGEM7Zf. RNA generated from the vector (pGEM) or the recombinant plasmid (GRIM-19), by *in vitro* transcription, was programmed into rabbit reticulocyte lysate and translated in the presence of 35S-methionine using the Ribo Max system (Promega Inc). Positions of the molecular weight markers (kDa) are shown on the right. (B) Silver stained gel of bacterially expressed GRIM-19 protein. GRIM-19 cDNA (GRM-19) was subcloned into pET32B vector (Tag) and expressed as a His-Tagged fusion protein. The recombinant protein was used for immunizing rabbits, after the removal of tag. Pre- and post immune sera were used for western blotting (1:2000 dilution) in panels (C) and (D), respectively.

**Figure 5:** Antisense inhibition of GRIM-19 gene expression. HeLa (panels A and B) and MCF-7 (panels C and D) cells stably transfected with pTKO1 (V) or pTKO1-GRIM-19 (G) as in figure 3 and were treated with IFN-β (500U/ml) and RA (2µM) for 5 days. RNA (30 µg) or protein (200 µg) extracted from the cells was used in the experiment. Panels A and C show the northern blot data and panels B and D show the western blot data. Letter E in panels A and C indicates the position of endogenous GRIM-19 mRNA. Arrow shows the position of transgene derived antisense mRNA. Note the ablation of protein expression in the antisense GRIM-19
transfected cells. Panel E is similar to panel D except that a monoclonal antibody against IFN inducible RNAseL was used in the experiment.

Figure 6: Induction of GRIM-19 gene expression by IFN-β and RA combination. Various breast carcinoma cell lines were treated with IFN-β (500U/ml) and RA (1μM), total RNA was extracted, and northern blot analysis was performed using a 32P-labeled GRIM-19 probe. Numbers above the blots indicate the hours at which RNA was extracted after IFN/RA treatment. At the bottom of each panel are the corresponding ethidium bromide-stained blots showing the presence of comparable amount of RNA in all lanes. Panel D shows the induction of GRIM-19 gene by various agents. RNA (30 μg) from MCF-7 cells was extracted after 2 days of treatment with indicated agents and used in northern blot analysis. Upper part of panel D shows the data obtained after probing with GRIM-19 cDNA. Lower part of panel D shows the same blot probed with a 32P-labeled β-actin cDNA. C: No treatment; I: IFN-β; IR: IFN-β/RA combination; and R: RA. Panel E: Induction of GRIM-19 protein by IFN/RA combination. Cells were labeled with 35S methionine during IFN/RA treatment and equal amount of protein from each sample was immunoprecipitated using pre-immune or post-immune IgG. The immunoprecipitates were separated on a 12% SDS-polyacrylamide gel and fluorographed. Plus (+) and minus (-) signs indicate IFN/RA or no treatment. Concentrations are same as in panel A-C. Numbers on the left indicate the positions of molecular weight markers. Panel F: Specificity of GRIM-19 antibodies. Pre- and post-immune IgGs were employed to immunoprecipitate GRIM-19 from a 35S-labeled cell extract. The position of the immunoprecipitated GRIM-19 band is shown with an arrow. Where indicated, the antibodies were preincubated with 5μg of bacterially expressed, purified recombinant GRIM-19 protein (rGRIM-19) for 1h at room temperature, prior to using in the immunoprecipitation. Panel G shows the effect of IFN-β, RA or their combination on the induction of GRIM-19 protein in MCF-7 cells. Cell extracts were prepared after treatment with various agents (as in panel D) and 150 μg of protein from each sample was western blotted. These blots were probed using the indicated antibody. Notations for each treatment are defined in panel D.

Figure 7: Tissue specific expression of the GRIM-19 in normal human tissues. Multiple tissue northern blots (Clonetech Inc) containing polyA+ mRNA (2μg per lane), derived from the
indicated human tissues, were probed with a $^{32}$P-labeled human GRIM-19 cDNA in panel A and C. Tissue source of the RNA was indicated above the tracks. Positions of the molecular weight makers were shown on the left of each blot. Panel B shows the same blot probed with a labeled $\beta$-actin cDNA. Two different actin mRNAs present in skeletal and cardiac muscles are indicated with filled and open triangles.

**Figure 8:** Death promoting effect of GRIM-19. (A) Overexpression of GRIM-19 in sense orientation causes a loss of cell viability. MCF-7 cells were transfected with equimolar amounts of mammalian expression vector pCXN2-myc (V) or the same vector carrying GRIM-19 (G). After 3 weeks of selection, with G418 (1mg/ml), the surviving colonies were counted. Each bar represents mean±SE of triplicates. (B) and (C) show the northern and western blot analyses, respectively, of the pooled cell clones derived after transfecting pCXN2 (V) and GRIM-19 cDNA (G). Arrow indicates the position of the mRNA in transgene expressing cells. Letter E indicates the endogenous mRNA. Panel D was probed with monoclonal antibodies specific to myc-tag. Signals in the vector lanes are not detectable because the peptide ran out of the gel. Panel D: Over expression of GRIM-19 enhances the cellular sensitivity to IFN/RA induced death. SRB binding assay was conducted with cells exposed to IFN-β (500U/ml), RA (1µM) or their combination for 4 days. Empty bars: Vector transfected cells; Filled bars: GRIM-19 transfected cells. Panel E: Annexin-V binding to the cells. Cells treated for 4 days with IFN-β/RA combination as above, prior to FACS analysis with FITC labeled annexin-V and propidium iodide. In Panel F, MCF-7 cells overexpressing the GRIM-19 gene were treated with indicated doses of caspase inhibitor z-VAD-fmk. They were then exposed to IFN/RA combination as above. Cells were processed for FACS analysis as described in panel E. Panel G: Higher caspase activity in GRIM-19 transfected cells. Vector or GRIM-19 expressing MCF cells were treated with the indicated agents as above. Cell lysates were prepared and caspase-9 activity was measured using a commercially available colorimetric kit (Biosource International Inc). Equal amount of total protein (50µg) was used in the assay. Absorbance was monitored at 405nm. Each bar represents mean±SE of triplicate samples. Panel H is a western blot using a commercially available anti-Caspase-9 antibody. Approximately 100 µg of total protein from each sample was western blotted.
Figure 9: GRIM-19 augments apoptotic death. HeLa cells were transfected with 1 µg of expression vector pCXN2 or the same vector carrying GRIM-19 cDNA using lipofectamine plus reagent. Cells were treated with indicated agents for 72h. They were then fixed and stained with DAPI (0.2µg/ml) and were observed under a Nikon fluorescent microscope. Typically ~30% transfection efficiency was obtained. Percentage of fragmented nuclei relative to total number of nuclei from multiple fields (n=24) was determined and plotted. A total of at least ~300 nuclei were counted from various fields. In Panel A, a representative field transfected with vector (on the left) or GRIM-19 (on the right) is shown. Magnification is 500X. Cells were treated with IFN-β (3000 U/ml), RA (5µM) or their combination where indicated. Panel B shows the effect of GRIM-19 on IFN/RA induced nuclear fragmentation. Panel C shows the effect of individual drugs on GRIM-19 induced cell death in an independent experiment. A background death value (11% from untreated cells) has been subtracted from the all the samples. Each bar represents mean percentage ± SE.

Figure 10: A mutant GRIM-19, lacking the C-terminus, does not augment IFN/RA induced death. Panel A: MCF-7 cells were transfected with a mutant GRIM-19 cDNA, GRIM-19Δ1, expressing as a myc-tagged protein. Extracts (300µg) from pCXN2 (Vector), wild type GRIM-19, and GRIM-19Δ1 were analyzed by western blotting with monoclonal antibodies specific to myc-tag. Numbers (kDa) on the left indicate the positions of molecular weight markers. Panel B: These cells were exposed to IFN/RA combination for determining their sensitivity using the SRB assay. Experiment was conducted as in Figure 1. Absorbance values for 0% and 100% growth in this experiment are vector: 0.158 & 0.757; GRIM-19: 0.152 & 0.710; and GRIM-19Δ1: 0.164 & 0.865, respectively.

Figure 11: Intracellular localization of GRIM-19. Permeabilized HeLa cells were fixed, incubated with rabbit polyclonal antibody specific for GRIM-19, washed and then incubated with Texas red-labeled anti rabbit IgG (A). These cells were also counter stained with DAPI (0.2µg/ml) to visualize the nuclei (D and E). Cells were photographed at 1000X magnification. Left panel: Texas-red staining; Right panel: DAPI staining of the same field. Panel C is similar to panel A, except that GRIM-19 antibody was incubated with recombinant human GRIM-19 for
1h prior to its use in the experiment. Note the blockade of Texas red staining in the bottom left panel.

**Figure 12:** Intracellular location of GRIM-19 does not alter significantly following exposure of cells to the drugs. HeLa cells were treated with IFN-β (3000U/ml), RA (5μM) or their combination for 4 days. Cells were stained with polyclonal antibodies specific for GRIM-19 and DAPI as described in Figure 11 and photographed. Note the DNA fragmentation and high staining of cell nucleus with GRIM-19 antibodies in the presence of IFN/RA combination. Top and bottom panels correspond to the same field. Specific treatments are indicated above the panel.
Figure 1
Figure 2

Figure 3: Sequence alignments
Figure 6
Figure 7
Figure 9
Figure 10

Figure 11
Identification of grim-19, a novel cell death regulatory gene induced by the interferon-beta and retinoic acid combination, using a genetic approach
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