

The Effect of Cytokines on Wild-type and Fanconi Mouse Embryonic Fibroblasts

I. Introduction

Fanconi's Anemia (FA) is an autosomal recessive chromosomal instability disorder. The symptoms include congenital abnormalities, defective hematopoiesis (formation of cells of the blood), and high risk of developing acute myeloid leukemia and solid tumors. The term autosomal refers to one of the 22 chromosomes that is not sex-linked and recessive means that the abnormal gene must be inherited from both parents. Tischkowitz and Dokal (2004) approximate the cumulative incidence of leukemia in FA patients by age 25 to be around 10% and the cumulative incidence of solid tumors to be around 29%. Grover Bagby, MD, is the OHSU Cancer Institute Director. His research is dedicated to unraveling the mysteries of cancer by researching Fanconi's Anemia. This rare disorder has a birth incidence of roughly 3 per million births (Tischkowitz and Dokal, 2004). The standard diagnostic test for FA is the chromosome breakage test, where a DNA cross-linking agent such as mitomycin C (MMC) or diepoxybutane (DEB) causes a higher level of chromosome breakage in Fanconi cells than normal cells.

Fanconi researchers have long been puzzled by how Fanconi Anemia (FA) cells, which are known to be more likely to perish under harsh conditions, somehow morph into cancer cells at an alarming rate since abnormalities usually cause cells to initiate apoptosis (programmed cell death). Some findings by Dr. Bagby's lab in murine ovarian tissue indicated that Fanconi cells were more sensitive to cytokines than wild-type cells of that tissue type. Therefore, preliminary studies exposing other cell types to cytokines

were deemed to be useful. Studies comparing the proliferation of wild-type and Fanconi cells after cytokine exposure were conducted. Background will be provided before discussing experiments and results in more detail.

II. Hypothesis

There is a tremendous amount of literature indicating that hypersensitivity to the alkylating agent mitomycin-C is a hallmark of Fanconi cells. Thus it is logical to suspect that Fanconi cells might be more sensitive (e.g. increased or decreased apoptosis/proliferation) to cytokines than wild-type cells.

The hypothesis of the experiments conducted is that Fanconi murine embryonic fibroblasts (MEFs) are more sensitive to cytokines than wild-type MEFs.

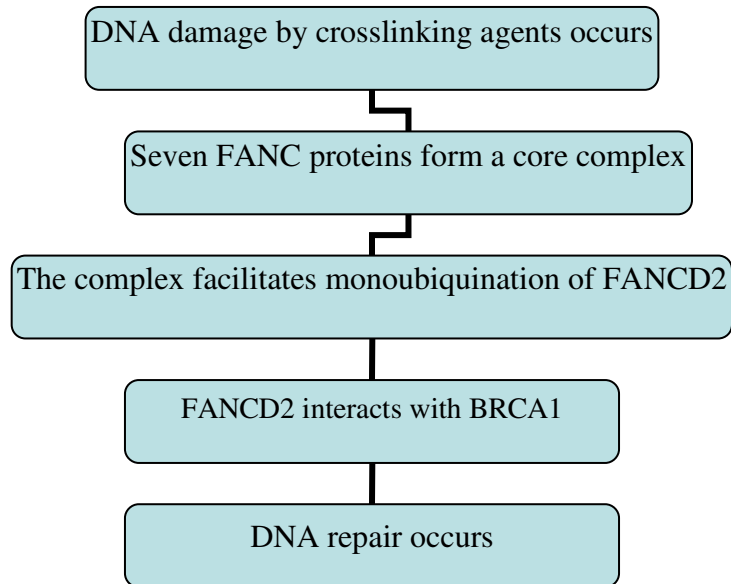


Figure 1: Example of how the molecular genetics of FA function: process thought to protect against chromosomal abnormalities.

Apoptosis

One characteristic of cancer cells is their ability to avoid planned death, which affects normal cells. This makes the study of cell death critical to understanding cancer.

Apoptosis is programmed cell death, and it is initiated either for developmental purposes (for example in the fetus the formation of the fingers and toes requires that the tissue in between digits be eliminated by apoptosis) or for the benefit of the entire organism (for instance when a cell's DNA is defective or it is infected by a virus). To prevent abnormalities from developing and causing harm to the organism, apoptosis effectively eliminates defective cells. This process also appears to be critical to balancing out cell proliferation. Apoptosis is distinguished from necrosis because it generally concerns

isolated cells versus groupings of cells. Apoptosis is more analogous to cell suicide than cell murder via physical circumstances such as changes in osmotic pressure or temperature changes (Potten and Wilson, 2004). Apoptosis functions so that the material from the dead cells is quickly processed. Cells undergoing apoptosis undergo condensation of chromatin and degradation of the nucleus, the cell itself then shrinks and breaks up into membrane-enclosed pieces, phosphatidylserine is exposed due to translocation, phagocytic cells bind phosphatidylserine and take in the cell pieces, finally the phagocytic cells secrete anti-inflammatory cytokines (Kimball, 2005).

Apoptosis Inducers

Cells are induced to initiate apoptosis by chemical signals. Signals operate through positive and negative feedback systems. Positive signals must be received continuously to perpetuate the life of the cell (ie: Interleukin-2), negative signals steer the cell toward apoptosis (ie: TNF-alpha and Fas Ligand).

Another way to categorize apoptosis inducers is by their source of origin. One of the means of inducing apoptosis is by external signals which bind to receptors on the outside of the cell, thus triggering actions by caspases, a group of cysteine proteases which perform a wide range of tasks in carrying out and regulating apoptosis. Three such external signalers are TNF-alpha, Fas Ligand (fibroblast-associated ligand, CD95L in humans), and interferon-gamma; these three cytokines were used in treatment of cells in the proliferation experiments.

A second class of apoptosis inducers, internal signaling, involves the mitochondria. A protein called Bcl-2 is present on the outer surface of the mitochondria; internal damage leads to the activation of another protein which makes holes in the mitochondrial membrane. Cytochrome c leaks from the mitochondrial holes, then binds to the apoptotic protease activating factor-1 (Apaf-1). These molecules come together and constitute apoptosomes; the apoptosomes activate caspase-9, initiating the caspase cascade (see Figure 2). The caspases degrade the remains of the cell before phagocytosis (Kimball, 2005).

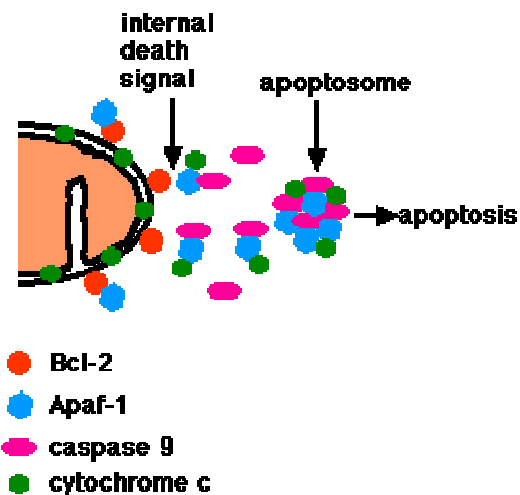


Figure 2: Illustration of apoptosis triggered by internal signals (Kimball, 2005)

TNF-alpha

TNF-alpha is a 17-kDa cytokine produced by T cells, mast cells and macrophages stimulated by LPS or IFN-gamma (Lin et al., 1996). Tissues affected by this molecule generally have the following symptoms of inflammation: heat, swelling, redness, and pain. In combination with other cytokines TNF-alpha can also induce one of the most

devastating symptoms associated with the end-stage of cancer: cachexia (loss of weight, muscle atrophy, weakness, and fatigue). Reportedly it also stimulates phagocytosis in macrophages and increases insulin resistance in some tissues.

TNF-alpha is at the root of everything known about cancer, it both suppresses and induces apoptosis. TNF-alpha has a long history as a cancer treatment. Pre-1900 Dr. William B. Coley treated cancer patients with a “crude bacterial filtrate” that is now known to have contained high concentrations of TNF-alpha. The treatment induced both fever and tumor necrosis in best-case scenario patients. In the 1970’s it was isolated from the serum of endotoxin-treated mice, allowing research at the molecular level to reveal the significance of this cytokine in immunology. A treatment which seeks to harness TNF-alpha’s therapeutic effects through local high-dosage is undergoing phase II clinical trials in patients with solid tumors. However, a mounting body of evidence shows that TNF-alpha may promote tumorigenesis when concentrations are low and sustained over time because it is not toxic in itself to tumor cells and acts as a growth factor in some types of tumors. This is not surprising, cancer has long been considered an inflammatory disease. Szlosarek et al. (2006) describes in detail the interactions between sustained low-levels of TNF-alpha, inflammatory cells, and tumor cells (747):

- Production of nitric oxide (NO) (DNA/enzyme damage, cGMP-mediated tumor promotion)
- Autocrine growth and survival factor for malignant cells
- Activation of E6/E7 mRNA in human papilloma virus (HPV)-infected cells
- Activation of Src kinase activity

- Tissue remodeling via induction of matrix metalloproteinases (MMPs)
- Control of leukocyte infiltration in tumors via modulation of chemokines and their receptors
- Down-regulation of E-cadherin, increased nuclear pool of beta-catenin
- Enhancement of tumor cell motility and invasion
- Epithelial-to-mesenchymal transition
- Induction of angiogenic factors
- Loss of androgen responsiveness
- Resistance to cytotoxic drugs

TNF-alpha is also associated with many symptoms of autoimmune diseases such as rheumatoid arthritis, ankylosing spondylitis, Crohn's disease, and psoriasis. Several drugs use monoclonal antibodies (Remicade and Humira) or receptor fusion proteins (Enbrel) to block the effects of TNF-alpha. These drugs are classified as immunosuppressants.

IFN-gamma

Interferon-gamma is a 25-kDa cytokine produced by T cells and natural killer (NK) cells (Lin, et al, 1996). It was named an interferon at first because of its observed antiviral effects. It also has significant antiproliferative and immunomodulatory effects. It is classified as a type II IFN due to the fact that its structure differs from type I IFNs.

IFN receptors exist on hematopoietic and non-hematopoietic cells. Signaling begins with the dimerization of the two subunits of the IFN-gamma receptor. In turn the receptor associated tyrosine kinases called Jak1 and Jak2, which through a signaling

cascade activates the JAK-STAT pathway (Strehlow and Schindler, 1997). Many cytokines have been found to activate this pathway which leads to the induction of certain genes. Research has found that mice which do not have the genetic material for producing IFN-gamma are very similar to those mice which lack the capability to make Stat1 (a signaling molecule in the JAK-STAT pathway). This evidence suggests that Stat1 is a common signal downstream of IFN-gamma.

According to Lin, et al. one of IFN-gamma's main roles along with LPS (lipopolysaccharide) is the differentiation of macrophages. This highly differentiated macrophage produces more IL-1 and TNF-alpha (1996).

Fas Ligand (FasL)

Fas Ligand is a type II transmembrane protein which is a member of the TNF family. It induces apoptosis by binding to three monomers of Fas receptor on cells where this receptor is present. This process is not a definite sign that apoptosis will occur.

Flow cytometry

Flow cytometry was the method used to analyze the treated cells in the experiments performed. Flow cytometry is a means to measure physical and chemical characteristics of cells in medium by sending a small stream of cells treated for the procedure past a light source induced by a laser, which is set to emit a certain wavelength of light as seen in Figure 3. A system of optics then collects the resultant absorbance or fluorescence

profile, which is then analyzed by software to determine sample statistics. Any cell component to which a fluorescent agent can be attached can be measured, making a wide range of assays possible. Some of the common applications of flow cytometry are phenotypic analysis, sterile sorting of transfectants, DNA analysis, functional studies, and apoptosis assays (“What is Flow”).

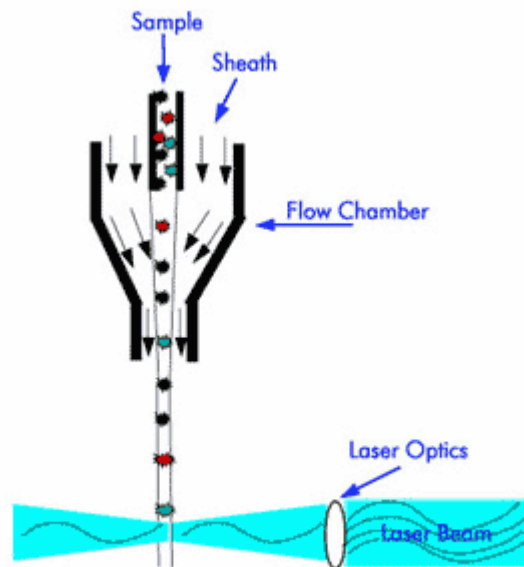


Figure 3: Flow Cytometer Schematic. (“Basic.”)

Flow cytometry was used in cell proliferation assays, as opposed to other cell proliferation assays which would be read with a fluorescence plate reader or contain radioactive particles. The assay used in this research project was the Guava Nexin™ Assay which has two main components: Annexin V-PE and 7-Aminoactinomycin D (7-AAD).

Annexin V is part of the family of proteins called annexins that all bind calcium and phospholipids. PE (phycoerythrin) is a fluorescent dye, a large protein, and is attached to Annexin V to indicate the presence of phosphatidylserine, for which it has a high affinity. This component differentiates between non-apoptotic cells and early/late-stage apoptotic and dead cells because early in the apoptotic pathway the phosphatidylserine molecules located on the internal face of the cell membrane are translocated to the external face of the membrane.

7-AAD is a fluorescent dye which is incapable of permeating the intact cell membrane and has a high affinity for DNA. It is useful for differentiating between cells whose membranes have begun breaking down and those whose membranes are severely compromised to the point that they do not enclose the DNA. By these two means, cells are sorted into three categories (see Figure 4.) (“The Guava Nexin™ Assay.”):

1. Non-apoptotic cells: Annexin V(-) and 7-AAD(-)
2. Early apoptotic cells: Annexin V (+) and 7-AAD(-)
3. Late stage apoptotic and dead cells: Annexin V(+) and 7-AAD(+)

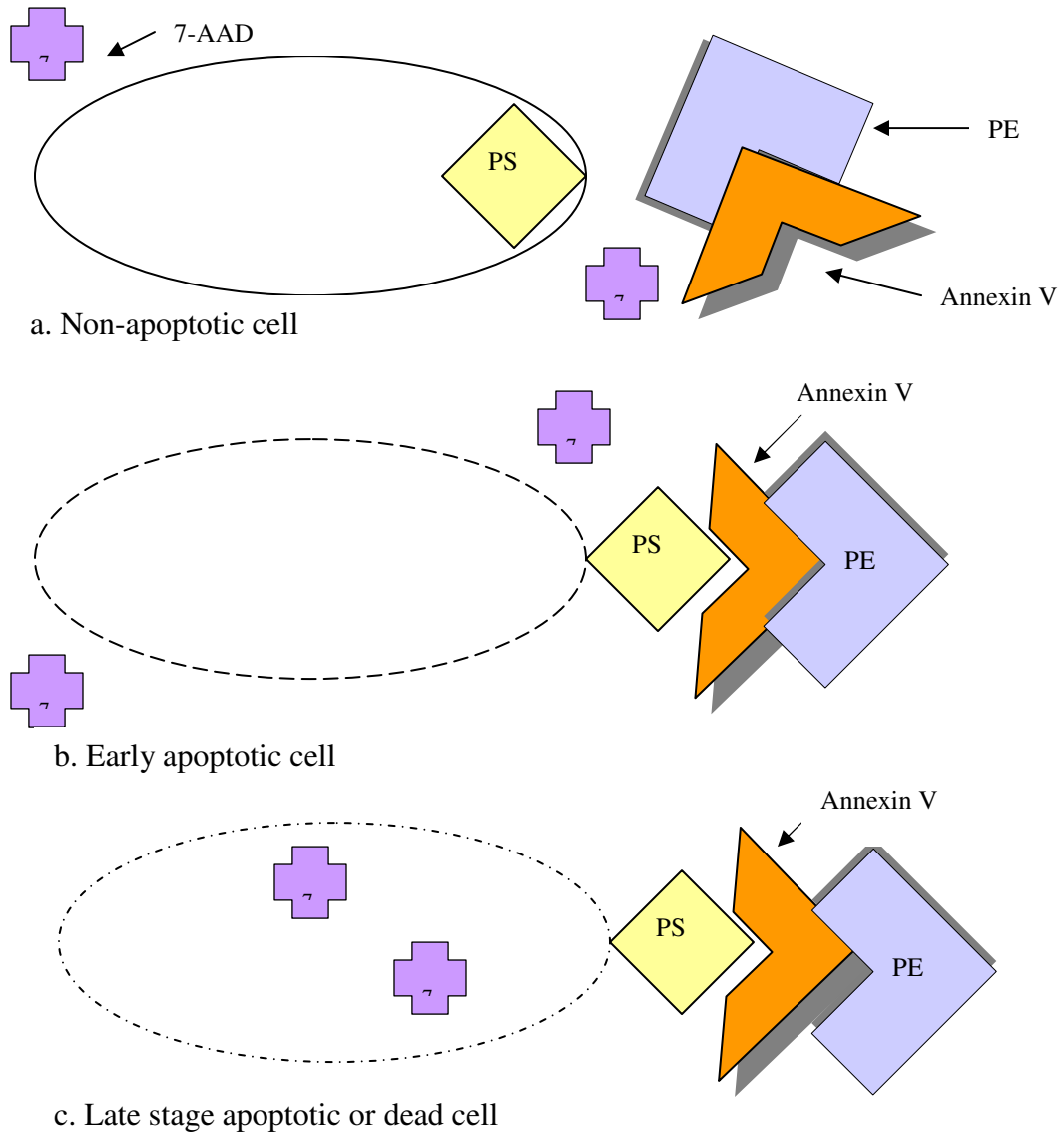


Figure 4: Schematic of the Guava Nexin™ Assay mechanisms.

IV. Materials and Methods

Cells

The cells used in these assays were murine embryonic fibroblasts (MEFs). Mouse cells were used because human Fanconi cells would be very difficult to obtain due to the low frequency of occurrence of Fanconi's Anemia. MEFs were selected for experimentation because they are relatively easy to maintain, are an established research model and are considered primary cells (versus immortalized cells which are more likely to have characteristics which differ from in vivo cells). Fibroblasts also affect cancerous regions because they are a component of tumor stroma, which is the environment near a tumor which interacts with and supports it. The cells are from a mouse line developed by a Dr. Buchwald and called "Buchwald Fanconi C57 black 6", (notated FancC -/- C57Bl/6). All the wild-type cells used are from the same cell line (ie: same embryo). All the knock-out cells used are from the same embryo, but from different splits (different splits means from different flasks of the same original group of cells).

Well-seeding

Cells were thawed from deep freeze temperatures and brought up a reasonable confluency in Dulbecco's Modified Eagle Media (10% FCS 1x P/S added) Petri dishes at 37 °C in a Forma Scientific CO₂ Water-Jacketed Incubator for approximately 3-7 days. The cell confluency was then determined by hemocytometer and the cells were divided into more Petri dishes so that in 24 hours the confluency of the plate would be high

enough to divide the cells into 24-well plates to be treated and analyzed by flow cytometry (concentrations of less than 15 cells/ μL were problematic because the flow cytometer is not designed for this low of concentration).

Exposure to Apoptosis Inducers

The treatments of cytokines were delivered by pipet into the microplate wells in solutions diluted to the desired concentrations in small quantities of media (<10 μL) the designated number of hours before being tested in the flow cytometer. The flow cytometer used was a Guava® PCA TM-96 System built by Guava Technologies, Inc. The cytokines used were mouse TNF-alpha, IFN-gamma, and FAS.

Annexin V Assay Protocol

Media was removed from cytokine-treated cells, then washed once with 1x PBS and trypsinized with 50 μl of 1x TE at 37 °C for approximately 5 minutes. During these five minutes, master mix was prepared by the formula of 126 μl dH₂O, 14 μl 10x Annexin V Staining Buffer, 5 μL Annexin V antibody, and 5 μl 7-AAD per sample (components from BD Pharmingen Annexin V-PE Apoptosis Detection Kit I). 150 μl of master mix was added to each sample using the multi-channel pipette. After adding the master mix, the samples were then incubated for 20 min in a dark environment at room temperature. In the meanwhile, the computer was turned on to program the worklist and the flow cytometer was turned on to warm the laser for 15 minutes (checking the “mix sample” option). First, the Nexin software was opened and a worklist started. The

worklist created from the file was selected and the 96 well-plate containing samples were loaded. [The 6 tubes for cleaning were filled with the fluids specified in the manual.]

The file in which data was recorded was named to save results.

Five main sets of experiments were conducted over a period of two months to test the effects of different cytokines, exposure over varying time periods, and to compare the effects of treatments on classes of cell types. The cytokines used to treat the cells were TNF-alpha, IFN-gamma, and FAS. The time intervals tested were between two and forty-eight hours. Cells were either wild-type or from Fanconi protein C knock-out mice. The treatments are summarized in Table I

Table I. Summary of Experiments

Date Experiment Identified By	Cytokine(s) Used	Cell Type(s)	Treatments	Time Intervals (hr)
7/9/05	TNF-alpha	WT	1. Control 2. 50 ng/mL TNF-alpha 3. 100 ng/mL TNF-alpha	2, 4,16, 24, 40, 48
7/15/05	TNF-alpha IFN-gamma	WT	1. Control 2. 20 ng/mL TNF-alpha + 0.4 U/mL IFN-gamma 3. 40 ng/mL TNF-alpha + 0.8 U/mL IFN-gamma	2,4, 6,16, 24, 40, 48
7/20/05	TNF-alpha IFN-gamma FAS	WT	1. Control 2. 20 ng/mL TNF-alpha + 200 U/mL IFN-gamma (T1) 3. 50 ng/mL TNF-alpha + 200 U/mL IFN-gamma (T2) 4. 20 ng/mL TNF-alpha + 200 U/mL IFN-gamma + 4 ug/mL FAS (T3)	6, 16, 24, 40, 48
7/28/05	TNF-alpha IFN-gamma FAS	WT KO	1. Control 2. 20 ng/mL TNF-alpha + 200 U/mL IFN-gamma + 4 ug/mL FAS	16, 24, 40, 48
8/10/05	TNF-alpha IFN-gamma FAS	WT KO	1. Control 2. 20 ng/mL TNF-alpha + 200 U/mL IFN-gamma + 4 ug/mL FAS	24

Note: all trials were done in triplicate, meaning 3 microplate wells were given each treatment.

V. Results and Discussion

A. Treatment I: TNF-alpha only

This treatment appears not to induce apoptosis at the levels of 50 or 100 ng/mL as seen in Fig. 4A. In fact, it seems to stimulate growth slightly; this affect has been reportedly induced by TNF-alpha before (Szlosarek, et al., 2006).

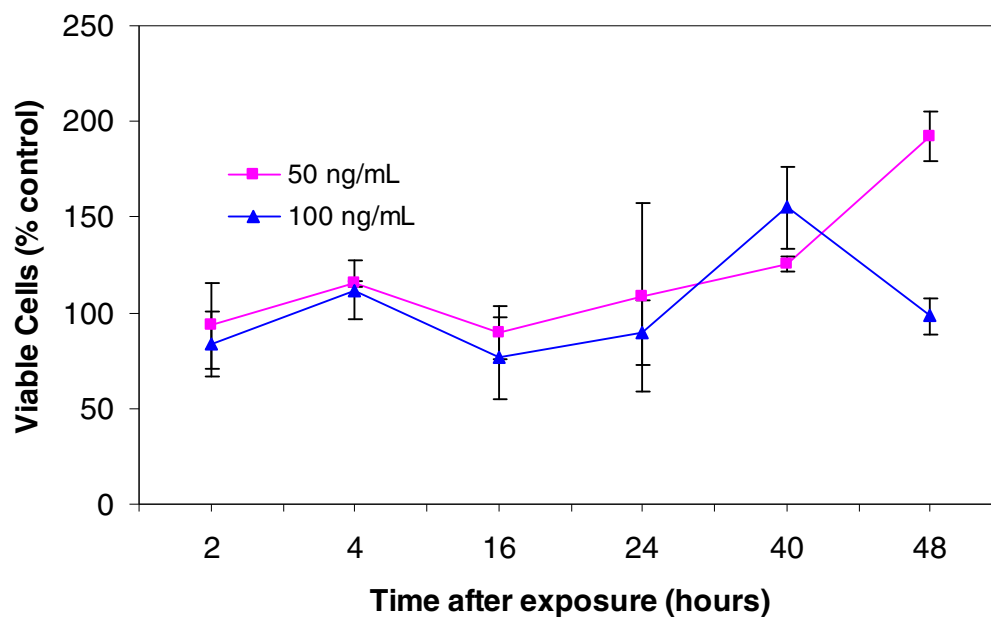


Figure 5A: Experiment (performed 7-9-05) exhibiting the effects of Treatment I

B. Treatment II: TNF-alpha & IFN-gamma

This treatment was tried at 4 levels of intensity:

- 20 ng/mL TNF-alpha + 0.4 U/mL IFN-gamma (not shown),
- 40 ng/mL TNF-alpha + 0.8 U/mL IFN-gamma (not shown),
- 20 ng/mL TNF-alpha + 200 U/mL IFN-gamma (T1 in Fig. 4B), and
- 50 ng/mL TNF-alpha + 200 U/mL IFN-gamma (T2 in Fig. 4B).

The first two dosages were so low in IFN-gamma that the effect was very similar to treating only with TNF-alpha (not shown). The highest concentrations applied showed a slight decrease in growth compared to the control, while the second highest showed a regular growth pattern (see Fig. 5B).

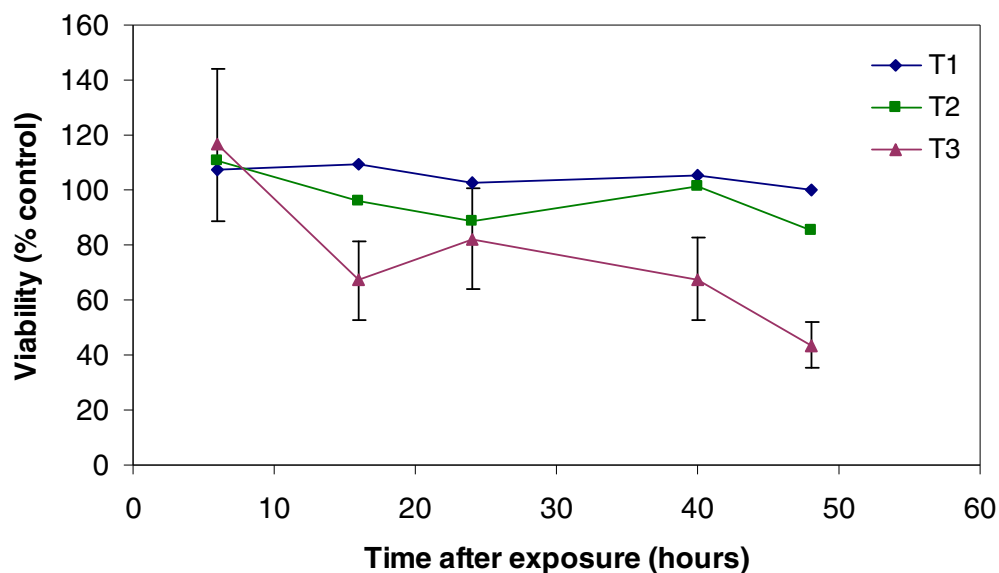


Figure 5B: Experiment (performed 7-20-05) exhibiting the effects of Treatment II in lines T1 and T2. T3 consists of 20 ng/mL TNF-alpha + 200 U/mL IFN-gamma + 4 μ g/mL FAS.

C. Treatment III: TNF-alpha, IFN-gamma, & FAS

This treatment (20 ng/mL TNF-alpha + 200 U/mL IFN-gamma + 4 µg/mL FAS) most reliably resulted in decreased viability. The “T3” line in Fig. 5B, the KO line in Fig. 5C, and both WT (wild-type) and KO (knockout) in Fig. 5D were all treated with the treatment mentioned above.

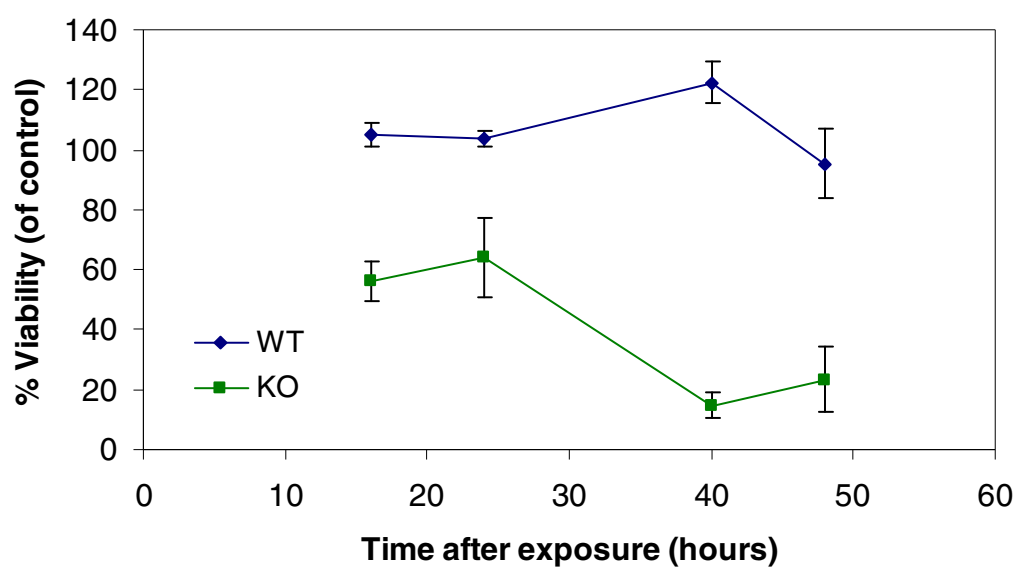


Figure 5C: Experiment (performed 7-28-05) exhibiting the effects of Treatment III in wild-type and knockout MEFs.

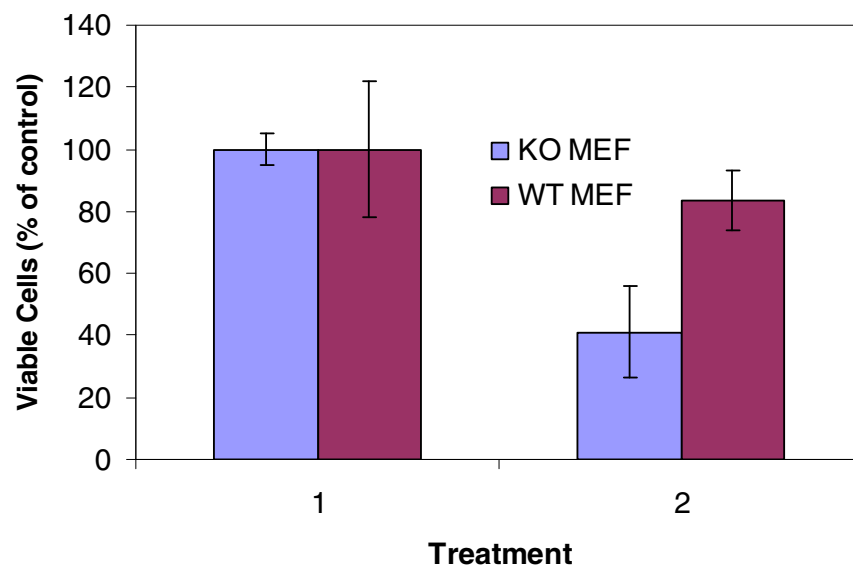


Figure 5D: Experiment (performed 8-10-05) exhibiting the effects of Treatment III in wild-type and knockout MEFs.

VI. Conclusions

These results suggest that FAS has an effect on MEF Fanconi cells and that Fanconi cells are more likely to induce apoptosis when exposed to cytokines than wild-type cells. It is possible that increased apoptosis was not seen in the wild-type cells in Fig. 5C(III) because the FAS added was not from a fresh solution. This problem was addressed in the subsequent experiment (Fig. 5D(IV)) and results were then similar to the Fig. 5B(II) graph when comparing the wild-type cells. These findings are significant because they suggest there is merit in testing the effects of cytokines on other Fanconi cell types.

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