

# Effect of a Nutrient Mixture on Fanconi Anemia Fibroblast and Normal Human Dermal Fibroblast: A Comparison

## Mohd Waheed Roomi, Tatiana Kalinovsky, Aleksandra Niedzwiecki, Matthias Rath

Dr. Rath Research Institute, Santa Clara, CA, USA Email: author@drrath.com

Received 18 February 2016; accepted 21 March 2016; published 24 March 2016

Copyright © 2016 by authors and Scientific Research Publishing Inc. This work is licensed under the Creative Commons Attribution International License (CC BY). http://creativecommons.org/licenses/by/4.0/

© Open Access

## Abstract

Fanconi anemia (FA) is a fatal heterogeneous autosomal recessive disorder, characterized by progressive bone marrow failure, congenital defect and cancer predisposition. Cell culture from FA fibroblast (FAF) displays certain abnormalities as compared to normal human dermal fibroblast (NHDF). This prompted us to investigate the effect of a specific nutrient mixture (NM) containing ascorbic acid, lysine, proline and green tea extract, which has demonstrated a broad spectrum of pharmacological activities, on FAF compared to NHDF. We investigated the in vitro effect of NM on FAF and NHDF cell proliferation by MTT assay, MMPs secretion by zymography, morphology by H&E staining and apoptosis by green caspase assay. FAF (FA-A: PD20, FA-A: PD220) and NHDF were cultured in modified Dulbecco Eagle media. At near confluence, the cells were treated with different concentrations of NM (0, 50, 100, 250, 500 and 1000 µg/ml) in triplicate. The cells were also treated with PMA to induce MMP-9 activity. NM had no effect on FAF cell viability in both cell lines compared to control. In contrast NM exhibited 20% at 50 and 100, 50% at 250, 60% at 500 and 70% toxicity at 1000 µg/ml on NHDF cells. Zymography demonstrated MMP-2 and MMP-9 on PMA stimulation in FAF and NM inhibited the activity of both MMP-2 and MMP-9 in a dose response fashion with total block at 500 µg/ml. In contrast, NHDF exhibited only MMP-2, both active and inactive forms, and NM inhibited their activities in a dose-dependent manner with total block at 1000 µg/ml. H&E staining did not indicate any morphological changes in FAF nor induced apoptosis at higher concentrations, as seen by caspases assay. However, although no morphological changes in NHDF were noted up to NM 100 µg/ml, progressive changes in cell shrinkage, rounding and nuclear condensation, pertaining to apoptosis, were observed at higher concentrations. These changes were consistent with the results from the green caspases apoptosis assay. Our data demonstrate that NM exhibited different responses toward FAF and NHDF. This may in part be due to elevated chromosomal break, deletion and hypersensitivity to cross linking agents, a DNA repair disorder in FAF that is lacking in NHDF.

How to cite this paper: Roomi, M.W., Kalinovsky, T., Niedzwiecki, A. and Rath, M. (2016) Effect of a Nutrient Mixture on Fanconi Anemia Fibroblast and Normal Human Dermal Fibroblast: A Comparison. *Open Journal of Apoptosis*, **5**, 1-8. http://dx.doi.org/10.4236/ojapo.2016.51001

#### **Keywords**

Fanconi Anemia Fibroblasts, Normal Human Dermal Fibroblasts, Nutrients, Cell Viability, MMP-2 and 9, Apoptosis

#### **1. Introduction**

Fanconi anemia (FA) is a heterogeneous autosomal recessive disease characterized by congenital abnormalities, deficiency of all three cellular components of the blood (red cells, white cells, and platelets), and an increased incidence of cancer. FA fibroblasts differ from normal human dermal fibroblasts, because they display elevated spontaneous chromosomal breaks and deletions and nuclear extracts that have substantially decreased plasmid-rejoining activity in contrast to normal fibroblasts [1] [2]. This prompted us to investigate the effect of a novel nutrient mixture (NM) containing ascorbic acid, lysine, proline and green tea extract, which has demonstrated a broad spectrum of pharmacological activities [3] on FAF compared to NHDF.

## 2. Methods and Materials

## 2.1. Fibroblast Cell Lines and Culture

Human FA fibroblast cell lines A:PD20 and A:PD220 were obtained from Fanconi Anemia Research Fund, Oregon Health & Science University, Portland, Oregon, USA). NHDF were obtained from ATCC. FAF (FA-A: PD20, FA-A: PD220) and NHDF were cultured in modified Dulbecco Eagle media supplemented with 20% FBS, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycinin 24-well tissue culture plates (Costar, Cambridge, MA).Cells were incubated with 1 ml of media at 37°C in a tissue culture incubator equilibrated with 95% air and 5% CO<sub>2</sub>. At near confluence, the cells were treated with NM, dissolved in media and tested at 0, 50, 100, 250, 500 and 1000  $\mu$ g/ml, in triplicate at each dose. Phorbol 12-myristate 13-acetate (PMA), 100 ng/ml was added to cells to induce MMP-9 secretion. The plates were then returned to the incubator. The media and sera used were obtained from ATCC, and antibiotics (penicillin and streptomycin) were from Gibco BRL, Long Island, NY.

#### 2.2. Composition of the Nutrient Mixture

The nutrient mixture (NM) was composed of the following in theratio indicated: Vitamin C (as ascorbic acid and as Mg, Ca, and palmitate ascorbate) 700 mg; L-lysine 1000 mg; L-proline 750 mg; L-arginine 500 mg; N-acetyl cysteine 200 mg; standardized green tea extract (derived from green tea leaves, was obtained from US Pharma Lab; the certificate of analysis indicated the following characteristics: total polyphenol 80%, catechins 60%, epigallocatechin gallate (EGCG) 35%, and caffeine 1.0%); 1000 mg; selenium 30 µg; copper 2 mg; manganese 1 mg.

## 2.3. MTT Assay

Cell viability was evaluated by MTT assay, a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] (MTT) to ablue formazan crystal by mitochondrial succinate dehydrogenase activity of viable cells. This test is a good index of mitochondrial activity and thus of cell viability. After 24 h incubation, the cells were washed with phosphate buffered saline (PBS) and 500  $\mu$ l of MTT (Sigma #M-2128) 0.5 mg/ml in media was added to each well. After MTT addition (0.5 mg/ml) the plates were covered and returned to the 37°C incubator for 2 h, the optimal time for formazan product formation. Following incubation, the supernatant was carefully removed from the wells, the formazan product was dissolved in 1 ml DMSO, and absorbance was measured at 570 nm in Bio Spec 1601, Shimadzu spectrometer. The OD<sub>570</sub> of the DMSO solution in each well was considered to be proportional to the number of cells. The OD<sub>570</sub> of the control (treatment without supplement) was considered 100%.

## 2.4. Gelatinase Zymography

Gelatinase zymography was performed in 10% Novex Pre-Cast SDS Polyacrylamide Gel (Invitrogen Corpora-

tion) in the presence of 0.1% gelatin under non-reducing conditions. Culture media (20  $\mu$ l) were mixed with sample buffer and loaded for SDS-PAGE with tris glycine SDS buffer, as suggested by the manufacturer (Novex). Samples were not boiled before electrophoresis. Following electrophoresis the gels were washed twice in 2.5% Triton X-100 for 30 minutes at room temperature to remove SDS. The gels were then incubated at 37°C overnight in substrate buffer containing 50 mM Tris-HCl and 10 mM CaCl<sub>2</sub> at pH 8.0 and stained with 0.5% Coomassie Blue R250 in 50% methanol and 10% glacial acetic acid for 30 minutes and destained. Upon renaturation of the enzyme, the gelatinases digested the gelatin in the gel, producing clear bands against an intensely stained background. Protein standards were run concurrently and approximate molecular weights were determined by plotting the relative mobilities of known proteins.

#### 2.5. Morphology and Apoptosis

Morphology of cells cultured for 24 h in test concentrations of NM were evaluated by H&E staining and observed and photographed by microscopy. At near confluence, FAF and NHDF cells were challenged with NM dissolved in media at 0, 100, 500, and 1000  $\mu$ g/ml and incubated for 24 h. The cell culture was washed with PBS and treated with the caspase reagent as specified in the manufacturer's protocol (Molecular Probes Image-IT<sup>TM</sup> Live Green Poly Caspases Detection Kit 135104, Invitrogen). The cells were photographed under a fluorescence microscope and counted. Green-colored cells represent viable cells, while yellow orange represents early apoptosis and red late apoptosis.

#### 2.6. Statistical Analysis

The results were expressed as means  $\pm$  SD, as indicated in the results, for the groups. Data was analyzed by independent sample "t" test.

## **3. Results**

#### 3.1. Cytotoxicity

NM exhibited no effect on FAF cell viability/proliferation, measured by MTT, in both cell lines compared to control, as shown in Figure 1(a), Figure 1(b). In contrast, NM showed inhibitory effects on NHDF cells, displaying 18.5% (p = 0.003) toxicity at 50 and 100 µg/ml compared to control and 33.3% (p = 0.0004) at 500 and 1000 µg/ml on NHDF cells, as shown in Figure 1(c).

## 3.2. Gelatinase Zymography

Zymography demonstrated MMP-2 and induction of MMP-9 with PMA stimulation in both FAF cell lines. NM inhibited the activity of both MMP-2 and MMP-9 in a dose response fashion with total block of both MMPs at 500 µg/ml. In contrast, NHDF exhibited only MMP-2, which was inhibited by NM in a dose-dependent manner with total block at 1000 µg/ml (see Figures 2-4).

## 3.3. Morphology (H&E Staining) and Apoptosis (Live Green Caspases Detection Kit)

H&E staining showed no morphological changes in the FAF (Figure 5) and slight apoptosis was observed at the highest concentration of NM (Figure 7), as shown in the caspases assay. Although no morphological changes in NHDF were noted up to NM 100  $\mu$ g/ml, progressive changes in cell shrinkage, rounding and nuclear condensation, pertaining to apoptosis, were observed at higher concentrations (Figure 6). These changes were consistent with the results from the green caspases apoptosis assay with almost total late apoptosis at 1000 NM (Figure 8).

## 4. Discussion

NM exhibited no effect on FAF cell viability in both cell lines compared to control. In contrast, NM demonstrated progressive dose-dependent toxicity to NHDF cells with 33% toxicity at 1000 µg/ml NM. These results were supported by the effects of NM on morphology and apoptosis in FAF compared to NHDF cells. H&E staining showed no morphological changes in the FAF and very slight apoptosis at the highest concentrations of NM. In contrast, morphology of NHDF demonstrated progressive changes in cell shrinkage, rounding and nuclear condensation, pertaining to apoptosis at concentrations higher than 100 µg/ml, which were consistent with







**Figure 2.** Effect of NM on MMP-2 and -9 secretion by FA PD:20 cells: Gelatinase zymography: (a) NM on FA PD:20 cells; (b) NM on PMA-treated FA PD:20 cells; Legend: 1—Markers, 2—Control, 3 - 7—NM 10, 50, 100, 500, 1000 µg/ml.



**Figure 3.** Effect of NM on MMP-2 and -9 secretion by FA PD:220 cells: Gelatinase zymography: (a) NM on FA PD:220 cells; (b) NM on PMA-treated FA PD:220 cells; Legend: 1—Markers, 2—Control, 3 - 7—NM 10, 50, 100, 500, 1000 µg/ml.



**Figure 4.** Effect of NM on MMP-2 and -9 secretion by NHDF cells: Gelatinase zymography: (a) NM on NHDF cells; (b) NM on PMA-treated NHDF cells; Legend: 1—Markers, 2—Control, 3 - 7—NM 10, 50, 100, 500, 1000 µg/ml.



Figure 5. Effect of NM on FA PD220 cell morphology: H&E. (a) Control; (b) NM 50 µg/ml; (c) NM 100 µg/ml; (d) NM 500 µg/ml; (e) NM 1000 µg/ml.



**Figure 6.** Effect of NM on NHDF cell morphology: H&E. (a) Control; (b) NM 50  $\mu$ g/ml; (c) NM 100  $\mu$ g/ml; (d) NM 500  $\mu$ g/ml; (e) NM 1000  $\mu$ g/ml.



Figure 7. Effect of NM on apoptosis of PA PD220 cells. (a) Control; (b) NM 100  $\mu$ g/ml; (c) NM 250  $\mu$ g/ml; (d) NM 500  $\mu$ g/ml; (e) NM 1000  $\mu$ g/ml.



**Figure 8.** Effect of NM on apoptosis of NHDF cells. (a) Control; (b) NM 100 µg/ml; (c) NM 250 µg/ml; (d) NM 500 µg/ml; (e) NM 1000 µg/ml.

the results from the green caspases apoptosis assay with almost total late apoptosis at 1000 NM. Liu *et al.* reported that Fanconi anemia cells demonstrate hypersensitivity to oxygen radicals, deficiency in antioxidant defense and increased production of reactive oxygen intermediates (ROIs) [4]. Ridget *et al.* hypothesized that the absence of functional proteins in FA lead to enhanced intracellular ROI levels implying that FA protein acts as an anti-apoptotoic protein via an antioxidant function [5].

In regards to invasive activity, zymography demonstrated MMP-2 and induction of MMP-9 with PMA stimulation in both FAF cell lines. NM inhibited the activity of both MMP-2 and MMP-9 in a dose response fashion with total block of both MMPs at 500 µg/ml. In contrast, NHDF secreted only MMP-2, which NM inhibited in a dose-dependent manner with total block at 1000 µg/ml. Epanchintsev *et al.* have demonstrated the overproduction of secretory factors such as IL-6, IL-8, MMP-2, and MMP-9 in FA, modulated by NF- $\kappa$ B/TNF- $\alpha$  signaling pathways, and showed that these over expressed secretory factors were effective in promoting the proliferation, migration, and invasion of surrounding tumor cells [6]. In studying MMP-9 secretion in varied cancer cell lines, we found that robust MMP-9 activity was associated with the most aggressive cancers [7]. Furthermore, experimental and clinical data has demonstrated an association between increased levels of MMP-9, and shortened patient survival, cancer progression and metastasis, since MMP-9 plays a significant role in tumor cell invasion and metastasis by digesting the basement membrane and components of the extracellular matrix [8]-[13]. NHDF is not invasive and secretes MMP-2 only. Invasion studies correlate with FA MMP results. A previous study of FA invasion through Matrigel showed that invasion was inhibited in FA PD:20 by 80% at 100 µg/ml and 100% at 500 µg/ml NM and in FA PD:220 by 96% at 50 µg/ml and 100% at 100 µg/ml NM [14].

## **5.** Conclusion

In conclusion, we found significant differences between the responses of Fanconi anemia fibroblasts and normal fibroblasts to NM treatment *in vitro*. The results showed MMP-9 secretion by FAF, not by NHDF, after PMA induction, which signifies an ability of cancer cell degradation of the extracellular matrix and mediator of cancer cell growth, invasion/metastasis and angiogenesis, explaining the predisposition of FA patients to cancer. NM significantly inhibited FAF MMP-9 secretion. NM exhibited no effect on FAF cell viability in both cell lines compared to control. In contrast, NM exhibited progressive dose-dependent toxicity to NHDF cells. These results were supported by the effects of NM on morphology and apoptosis in FAF compared to NHDF cells. The activities of FAF observed in this study may be due to the impaired DNA repair process with chromosomal break, deletion and hypersensitivity to cross linking agents found in FAF but not in NHDF.

# Acknowledgements

The research study was funded by Dr. Rath Health Foundation (Santa Clara, CA, USA), a non-profit organization.

### **References**

- Donahue, S.L. and Campbell, C. (2002) A DNA Double Strand Break Repair Defect in Fanconi Anemia Fibroblasts. Journal of Biological Chemistry, 227, 46243-46247. <u>http://dx.doi.org/10.1074/jbc.M207937200</u>
- [2] Donahue, S.L., Lundberg, R., Saplis, R. and Campbell, C. (2003) Deficient Regulation of DNA Double-Strand Break Repair in Fanconi Anemia Fibroblasts. *Journal of Biological Chemistry*, 278, 29487-29495. <u>http://dx.doi.org/10.1074/jbc.M213251200</u>
- [3] Niedzwiecki, A., Roomi, M.W., Kalinovsky, T. and Rath, M. (2010) Micronutrient Synergy—A New Tool in Effective Control of Metastasis and Other Key Mechanisms of Cancer. *Cancer Metastasis Review*, 29, 529-542. <u>http://dx.doi.org/10.1007/s10555-010-9244-1</u>
- [4] Ridet, A., Guillouf, C., Duchaud, E., Cundari, E., Fiore, M., Moustacchi, E. and Rosselli, F. (1997) Deregulated Apoptosis is a Hallmark of the Fanconi Anemia Syndrome. *Cancer Research*, **57**, 1722-1730.
- [5] Liu, J.M., Buchwald, M., Walsh, C.E. and Young, N.S. (1994) Fanconi Anemia and Novel Strategies for Therapy. Blood, 84, 3995-4007.
- [6] Epanchintsev, A., Shyamsunder, P., Verma, R.S. and Lyakhovich, A. (2015) IL-6, IL-8, MMP-2, MMP-9 are Overexpressed in Fanconi Anemia Cells Through a NF-κB/TNF-α Dependent Mechanism. *Molecular Carcinogenesis*, 54, 1686-1699. <u>http://dx.doi.org/10.1002/mc.22240</u>
- [7] Roomi, M.W., Kalinovsky, T., Rath, M. and Niedzwiecki, A. (2014) Effect of a Nutrient Mixture on Matrix Metalloproteinase-9 Dimers in Various Human Cancer Cell Lines. *International Journal of Oncology*, 44, 986-992.
- [8] Kleiner, D.E. and Stetler-Stevenson, W.G. (1999) Matrix Metalloproteinases and Metastasis. *Cancer Chemotherapy* and Pharmacology, **43**, S42-S51.
- [9] Yurchenko, P.D. and Schitny, J.C. (1990) Molecular Architecture of Basement Membranes. *The Federation of Ameri*can Societies for Experimental Biology Journal, 4, 1577-1590.
- [10] Barsky, S.H., Siegel, G.P., Jannotta, F. and Liotta, L.A. (1983) Loss of Basement Membrane Components by Invasive Tumors but Not by Their Benign Counterparts. *Laboratory Investigation*, 49, 140-147.
- [11] Liotta, L.A., Tryggvason, K., Garbisa, S., Hart, I., Foltz, C.M. and Shafie, S. (1980) Metastatic Potential Correlates with Enzymatic Degradation of Basement Membrane Collagen. *Nature*, 284, 67-68. <u>http://dx.doi.org/10.1038/284067a0</u>
- [12] Stetler-Stevenson, W.G. (2001) The Role of Matrix Metalloproteinases in Tumor Invasion, Metastasis and Angiogenesis. *Surgical Oncology Clinics of North America*, **10**, 383-392.
- [13] Stetler-Stevenson, W.G. (1990) Type IV Collagenases in Tumor Invasion and Metastasis. *Cancer Metastasis Reviews*, 9, 289-303. <u>http://dx.doi.org/10.1007/bf00049520</u>

[14] Roomi, M.W., Ivanov, V., Niedzwiecki, A. and Rath, M. (2005) Down Regulation of Matrix Metalloproteinases and Inhibition of Cell Invasion by a Novel Nutrient Mixture in Human FA Fibroblast Cell Lines: in Vitro Study. 17th Annual Fanconi Anemia Scientific Symposium Proceedings, Geneva, 29 September-2 October 2005, Abstract #110.