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IN SCHOOL ARTICLE

Vitamin D3 Blocks NFkB Activation in an In Vitro Model of Cystic Fibrosis

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Abstract

In this study, the inflammatory responses in isogenic lung epithelial cell lines derived from a patient with Cystic Fibrosis (CFBE41o- ΔF508 and CFBE41o- N) were examined. The first cell line overexpresses the ΔF508 deletion mutation leading to Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein dysfunction, while the second line overexpresses a normal allele of CFTR. Cells expressing the mutated CFTR protein have been shown to be more sensitive to inflammatory pathways mediated by the toll-like receptor 2 (TLR2)[1]. We hypothesized that CF cell lines would show an elevated inflammatory response when stimulated through this pathway and that this inflammatory response could be reduced by treatment with Vitamin D3. Vitamin D3 has been described to modulate TLR expression and is a known suppressor of the TLR2 and TLR4 pathways[2]. An inflammatory response was induced in the cells using MALP-2, a TLR2/6-specific ligand. Translocation of NFkB to the nucleus, as evidenced by immunofluorescent localization of nuclear NFkB, was used as an indicator of pro-inflammatory signaling. It was found that 16% of the cells expressing normal CFTR treated with MALP-2 showed NFkB nuclear translocation versus 28% of the ΔF508 cells treated with MALP-2. This almost two-fold increase in NFkB translocation strongly suggests that the ΔF508 cells may indeed be more sensitive to inflammation than the cells expressing normal CFTR. The potential role for Vitamin D3 in mitigating this inflammatory response was then examined. When the cells expressing normal CFTR were treated with MALP-2 and Vitamin D3, 5% of the cells expressed activated NFkB. When the same experiment was performed with cells expressing the mutation, 9% of the cells treated with MALP-2 and Vitamin D3 had translocated NFkB These results provide evidence for both the potential claim that ΔF508 cells are more sensitive to inflammation via TLR pathways and that Vitamin D3 does in fact reduce the inflammatory response in Cystic Fibrosis cells.

Introduction

Cystic Fibrosis (CF) is a recessive genetic disorder that results in frequent and severe lung infections and digestive problems. As a life threatening disease, CF affects the quality of life of many children and adults in the U.S[3] and is the most common lethal, autosomal recessive disease in Caucasians.[4]

CF is characterized by a mutation in the CFTR protein[5], which regulates the transport of chloride ions across the cell membrane of the lungs, liver, pancreas, digestive tract, reproductive tract, and skin. CFTR functions as a cAMP-activated and phosphorylation-regulated Cl channel, and it also regulates other membrane channels and/or proteins. Though many sequence variants have been found in the CFTR gene, most of which are associated with CF, the most frequent mutation in CF patients is a ΔF508 deletion mutation, accounting for about 66% of all CF alleles (See Figure 1).[6] The ΔF508 mutation is caused by a deletion of three base pairs that results in the loss of a phenylalanine at amino acid 508 in the protein.[4] This mutation prevents the protein from reaching the cell membrane. Studies have shown links between irregular CFTR protein and inflammation, the other major characteristic of CF.[7] The imbalance of ions, caused by poor chloride transport, creates a thick, sticky layer of mucus that traps bacteria (See Figure 2). The trapped bacteria lead to chronic inflammation in the airways of the affected patients.[8] Inflammatory pathways in lung epithelial cells are stimulated by ligands from the bacterial cell walls that bind to the TLR complexes.

TLR signaling is a key component of the innate immune response. Each TLR receptor recognizes specific ligands by pattern recognition. Once a TLR receptor binds its ligand, an inflammatory pathway becomes activated. There are many different types of TLR receptors that trigger many different pathways because they can bind different types of ligands. The receptors may act by themselves, or they may heterodimerize to form a combined TLR complex that recognizes even more ligands. TLR mediated responses are mostly controlled by the MyD88-dependent pathway. MyD88 is a protein that activates the transcription factor NFkB through the canonical signaling pathway (See Figure 3). The binding of the ligand to the TLR receptor leads to the recruitment of adaptors, such as TRAF6, leading to the release of inhibition of NFkB, which then allows NFkB to move into the nucleus.[9]

Prior to TLR activation, NFkB exists freely in the cytoplasm of the cell. When an inflammatory response is triggered, NFkB becomes activated, and it translocates from the cytoplasm to the nucleus, where it acts as a transcription factor. NFkB activates the transcription of genes coding for inflammatory cytokines such as interleukins (IL-6 and IL-8), important mediators of the inflammatory response that attract neutrophils to the target area, amplifying the inflammatory response.

Inflammation is an important biological function because it allows the body to fight against potentially harmful irritants.[10] While normal levels of inflammation lead to repairing and healing, exaggerated inflammatory responses can cause damage or harm (See Figure 4). In the case of CF patients, CFTR mutated cells are hypersensitive to inflammation caused by bacterial infection in the airways. This constant inflammation is one factor that leads to declined pulmonary function.[11]As described above, CF patients suffer through severe inflammation that greatly lowers

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their quality of life. If the inflammation in these patients could be controlled, then the distress caused by the disease could be alleviated. An inhibitor of inflammation could be used as a therapeutic treatment for these patients.

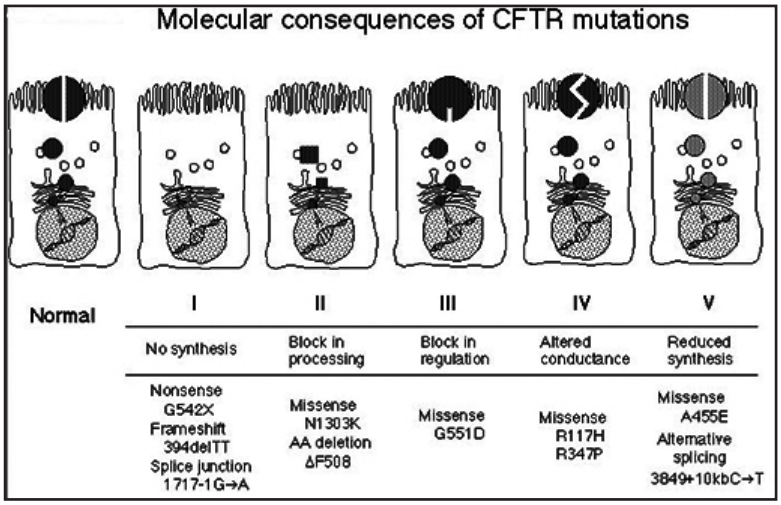

Figure 1. This figure shows some of the mutations that can result in CF. The effect of the mutation on the cell is also shown, ΔF508 is number two.[6]

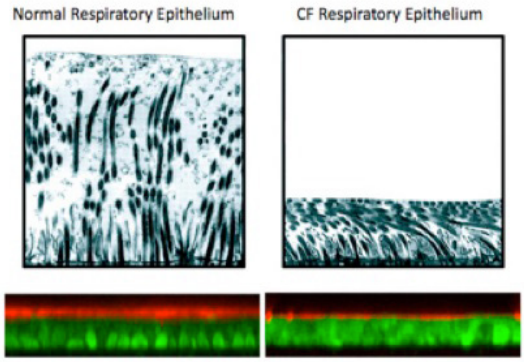

Figure 2: These images compare the respiratory epithelium in CF and normal bodies. CF results in failed transport of ions across the membranes. The top two pictures show the luminal airway surface. CF impairs the mucociliary escalator, which when functioning normally clean the airways and clear mucous to prevent infection. The normal ciliated airway epithelium is on the left and the CF is on the right. The pictures on the bottom compare the airway surface liquid layers (red), the green are the epithelial cells[17].

Materials and Methods

Cell Culture and Treatments: The two cell lines CFBE41o- N and CFBE41o- ΔF508 (kind gift from Dr. Dieter Gruenert, USCSF), were grown in culture flasks with MEM cell culture medium supplemented with 10% fetal bovine serum and 1x penicillin/streptomycin. The culture flaks had been pre-coated with a fibronectin solution containing 44 ml of LHC basal medium, 5 ml of bovine serum albium, ½ ml of collagen, and ½ ml of human fibronectin. For the cell lines stably transfected with the pCEP4 plasmid (CFBE41o- N, and CFBE41o- ΔF508) hygromycin B was added to the medium for continuous selection. The cells were passaged when they were between 80-100% confluent. A Hepes Buffered Saline (HBS) was used to rinse the cells prior to trypsinization.[13] For the experiments, the cells were plated at a target density of 20,000 cells per well in 12-well tissue culture plates each well containing a glass coverslip. These plates had been pre-coated in the same manner as the culture flaks. One ml of medium was added to each well. After 24 hours, the cells were treated for 30 minutes with a final MALP-2 (Imgenex) concentration of 50 ng/ml and/or a Vitamin D3 (Sigma Aldrich) concentration of 10^{-7}M.

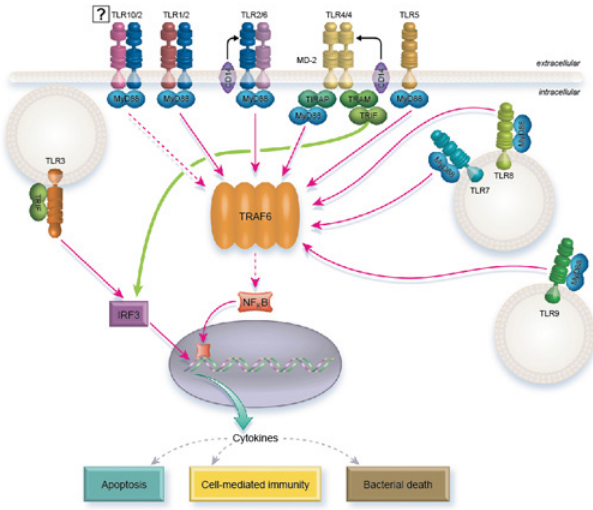

Figure 3: This figure shows the TLR signaling pathway. The TLR complexes are extracellular and once an agonist binds to the complex, the pathway is triggered and a transcription factor, such as NFkB, is translocated to the nucleus.[18]

Immunofluorescence, Microscopy: After treatment, cells were fixed with 4% paraformaldehyde/PBS for 15 minutes at room temperature and washed with PBS. The cells were then incubated in blocking solution (10% NDS, 0.1% Triton, PBS) for 30 minutes. The cells were then labeled with specific primary antibodies. For the NFkB translocation assay the cells were labeled with anti-NFkB rabbit IgG antibody (Santa Cruz, 1:100), diluted in blocking solution, overnight. After PBS washes, the cells were incubated in the secondary antibody/blocking mix for 30 minutes. For NFkB labeling, donkey anti-rabbit IgG- Dylight 549 (Santa Cruz, 1:100) was used. The cells were counterstained with Hoechst stain (1:100) and coverslipped with anti-fading mounting medium. The cells were observed using a Carl Zeiss Axioplan upright fluorescent microscope at a magnification of 200x. The images were captured using Openlab software. **Statistical Methods:** The significance (p-value) of the differences between the groups was estimated using a one-tailed Student's t-test. Two slides were counted for each condition for averaging the results.

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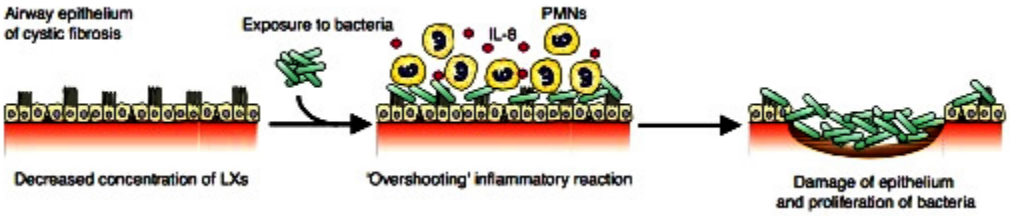

Figure 4: This figure show the damage that exaggerated inflammatory response may have on the airway epithelium of CF patients.[19]

Results

In this experiment, both the inflammatory responses of CF cells were studied in response to the TLR2/6 specific ligand MALP-2 as well as the anti-inflammatory properties of Vitamin D3 in the TLR2/6 pathway. The inflammation assays measured NFkB activation in the cells. Two different cell lines derived from a CF patient carrying two ΔF508 genes were studied; one line modified to express ΔF508 at closer to clinically observed levels, and one line expressing normal CFTR. The cells underwent specific treatments for 30 minutes prior to staining and microscopy. To measure the percent NFkB activated in the cells, the location of NFkB in the cell was stained with an antibody. Counts were made for three fields of view per slide and percentages were calculated (Figures 5 and 6). There were two slides per condition. The percentages were averaged in order to find the average percentage of NFkB activated for each condition. For each cell line there were four sets of conditions. The cells were either left untreated (-MALP-2 –Vitamin D3), treated with both (+MALP-2 +Vitamin D3), or treated with one or the other (+MALP-2 -Vitamin D3, -MALP-2 +Vitamin D3). Figure 7 shows a graphical representation of the data.

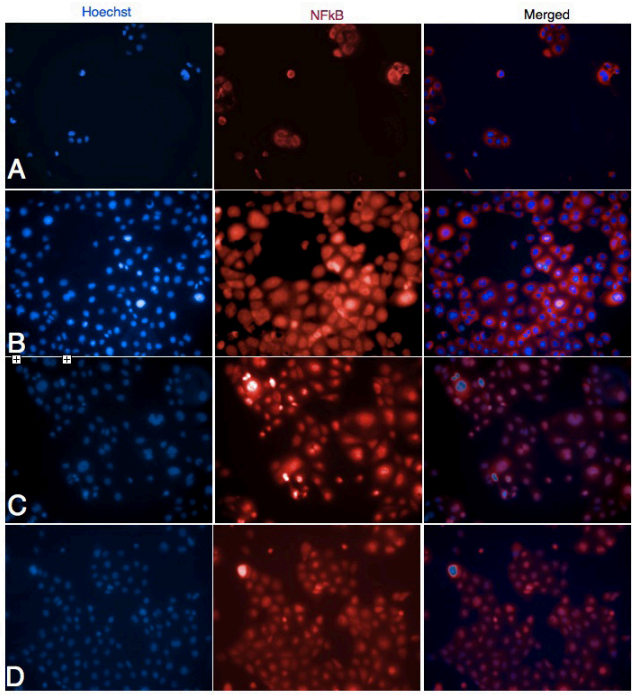

Figure 5: CFBEo- ΔF6.2 cells stained with Hoechst (blue) to reveal nuclei, and antibody to NFkB (red). (A) Cultured without MALP-2 and without Vitamin D3. (B) Cultured without MALP-2 and with Vitamin D3. (C) Cultured with MALP-2 and without Vitamin D3. (D) Cultured with MALP-2 and with Vitamin D3.

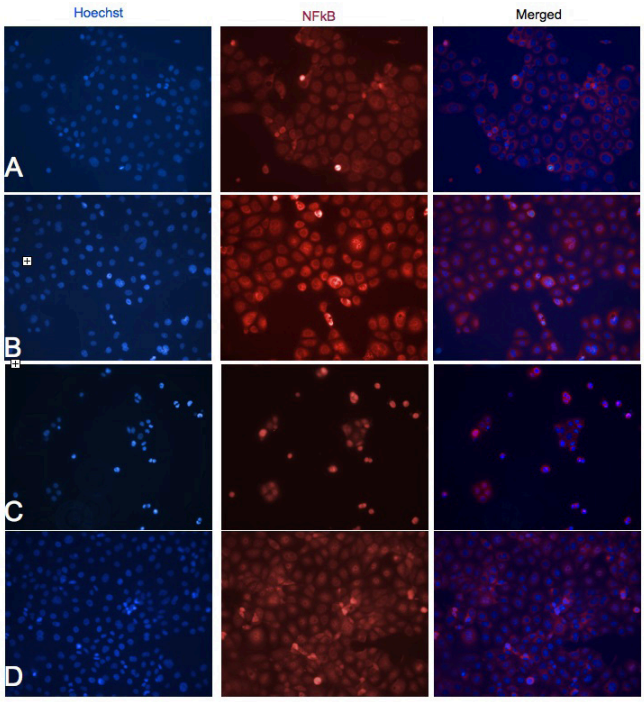

Figure 6: CFBEo- N6.2 cells stained with Hoechst (blue) to reveal nuclei, and antibody to NFkB (red). (A) Cultured without MALP-2 and without Vitamin D3. (B) Cultured without MALP-2 and with Vitamin D3. (C) Cultured with MALP-2 and without Vitamin D3. (D) Cultured with MALP-2 and with Vitamin D3.

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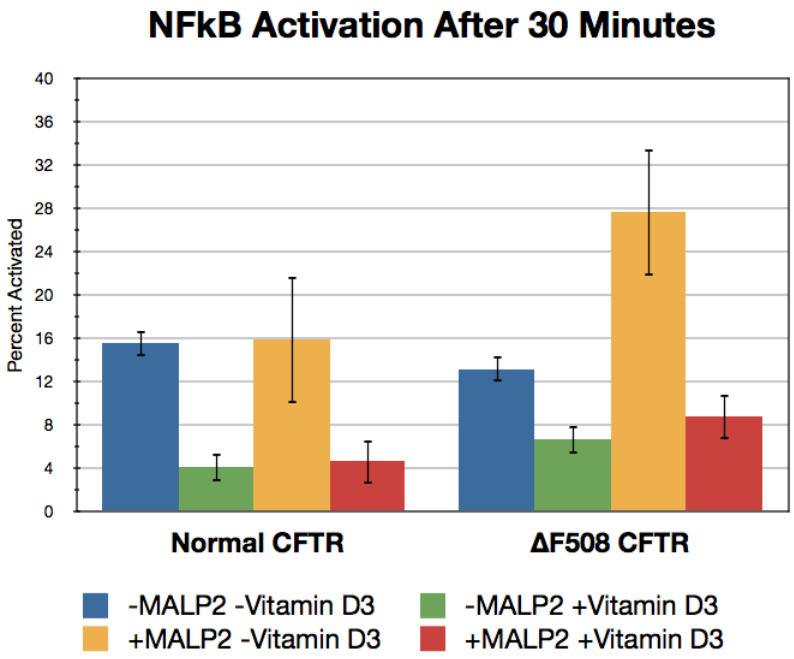

Figure 7: This figure shows the percentage of CFBEo- cells containing activated NFkB. Measures for cells expressing normal CFTR and cells with the ΔF508 mutation are represented for each condition.

When the ΔF508 cells were not treated, they showed an average activation of NFkB of 13%. When the ΔF508 cells were treated only with MALP-2 the average increased to 28%, a significant increase in response to the addition of low doses of MALP-2 ($p<0.02$). For the Vitamin D3 conditions it was found that when the ΔF508 were treated with Vitamin D3 alone, the amount of NFkB activated was 7% and when the cells were treated with both MALP-2 and Vitamin D3, the amount was 9%. The amount of NFkB activated when the ΔF508 cells were treated with both the MALP-2 and the Vitamin D3 showed a significant decrease from the amount of NFkB activated when only treated with MALP-2 ($p<0.003$).

The same tests were done on the cells expressing normal CFTR. When the cells expressing normal CFTR were not treated, they showed an average activation of NFkB of 15%. When they were treated with MALP-2, the percentage increased only marginally to 16%. This slight increase is not statistically significant ($p>0.4$). When Vitamin D3 was added to the cells expressing normal CFTR, it was found that Vitamin D3 alone decreased the percentage to 4%. When the cells were treated with both MALP-2 and Vitamin D3, the percentage went down to 5%, a significant decrease from the 16% when they were treated only with MALP-2 ($p<0.004$).

Discussion

In this study our objective was to explore the behavior of CFTR mutated cells. CF is a genetic disorder that greatly impacts the lives of those afflicted. Patients suffer from the discomfort of constantly inflamed air passages and bacterial infections. CF patients suffer from chronic inflammation, and as a result their quality of life is decreased.[14]

According to previous studies it has been speculated that one of the mutations that causes CF, ΔF508, also sensitizes the cells to inflammation.[15] There had also been indications that these cells would respond through the TLR2/6 inflammatory pathway. This study sought to examine this observation using two cell types. ΔF508 and cells expressing normal CFTR are both derived from a patient with the ΔF508 mutation, but the ΔF508 cells are complemented to express high levels of the dysfunctional protein and the cells expressing normal CFTR are complemented to express normal functioning copies of the protein. The first aim was to explore whether these cells responded thorough the pathway that we were targeting, TLR2/6, and also to see whether there were any behavioral differences between the two cell lines in response to the MALP-2 ligand.

It was hypothesized that, if the cells did in fact respond through this pathway, there would be a difference between the relative amount of ΔF508 cells that showed signs of inflammation and the relative amount of cells expressing normal CFTR that showed signs of inflammation. Because the ΔF508 cells express high levels of the mutated CFTR protein, these cells should be more sensitive to an inflammatory response when treated with a ligand. In order to quantify the inflammatory response, we stained for the location of NFkB as an indicator of inflammation. Our data show that the ΔF508 did have a higher response to the ligand than the cells expressing normal CFTR, as there were relatively more ΔF508 cells that showed translocated NFkB. The minimal increase in NFkB positive cells following stimulation with MALP-2 of the cells with a normal CFTR, and the contrasting and striking response observed in the ΔF508 cells, strongly

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suggests that the cells with the deletion are far more responsive to an inflammatory response induced by a ligand of the TLR2/6 pathway than are normal cells. Downstream effectors of inflammation such as Il-6 (Data Not Shown) were also explored. Although limited data was collected to allow firm conclusions, these data also suggest an increase in response to MALP-2. Nevertheless, the NFkB data alone are a strong indicator that these cells respond through this pathway. This insight into the behavior of cells that have the ΔF508 mutation may be a starting point into understanding possible treatments to help CF patients.

The second aim of this study was to examine the anti-inflammatory properties of Vitamin D3. As Vitamin D3 had been shown to inhibit the inflammatory response in certain TLR pathways such as TLR2 and TLR4, it was hypothesized that it might act as an anti-inflammatory complex in the TLR2/6 pathway as well, but there was no previous literature to support this claim.[9] As we did discover that both the ΔF508 and the cells expressing normal CFTR responded through the TLR2/6 pathway we aimed to perform a similar experiment using the MALP-2 to induce inflammation, but this time Vitamin D3 was added to some of the cells as well. If Vitamin D3 were to, in fact, inhibit the inflammatory response, a significant decrease in the amount of NFkB translocated to the nucleus should have been seen when the cells were treated with Vitamin D3 along with MALP-2, compared to when the cells were only treated with MALP-2. These results are consistent with the hypothesis. Treatment with Vitamin D3 significantly reduced the amount of NFkB that was seen translocated to the nucleus when inflammation was induced in the cells. These results very strongly suggest that Vitamin D3 treatment does act to inhibit the significantly elevated inflammatory response observed in ΔF508 cells after stimulation through the TLR2/6 pathway with MALP-2.

Even though these results indicate that Vitamin D3 is inhibiting inflammation, claims about the specific mechanism through which Vitamin D3 is acting cannot be made. As mentioned earlier, Vitamin D3 can inhibit the inflammatory response in two ways. It can either down regulate the expression of the TLR complexes on the membrane of the cell, or it can directly inhibit NFkB from translocating into the nucleus to continue the response.[2] Inferences about the mechanism can be made, though, as all the treatments performed in this study were of 30 minutes. If Vitamin D3 were down regulating the expression of the TLR complexes, 30 minutes would likely not be enough time to see any significant effects.[16] But, a significant decrease in the activation of NFkB was indeed seen when the Vitamin D3 was added to the cells. Therefore it is very likely that Vitamin D3 is actually inhibiting NFkB from moving into the nucleus, a response that would likely be observed in the span of 30 minutes. An obvious next step would be to examine the expression of the TLR2/6 complex on the cell membrane prior to treatment with Vitamin D3 and after the treatment. This would provide more insight into the mechanism through which Vitamin D3 is inhibiting inflammation in these cells.

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