Role of immunoregulators as possibility of tumor hypoxia-induced apoptosis

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ABSTRACT

IL-1, TNF-α and INF-γ cytokines stimulate in nitric oxide production and result with inflammation manifested by apoptosis and hypoxia. We propose that extent of inflammation leading to hypoxia initially and programmed cell death later both can enhance the MRI visible edema fluid content in breast and lung tumors due to oxygen and energy insufficiency to surviving inflammatory tumor epithelial cells. The mechanism of A549 cell damage due to NO production included: Cytokines regulated NO synthase regulation through NF-kB cytosolic factor; Cofactor tetrahydrobiopterin-catalyzed synthesis of iNOS (inducible nitric oxide synthase. The NO production and increased NO synthase lead to Na+ ion transport and cell proliferation with differentiation or apoptosis. Our recent report on hypoxia-induced apoptosis and decreased cell proliferation with differentiation supported the possibility of MRI as potential technique in alveolar and tumor imaging of inflammation [1]. Hypoxic cells showed the possibility of NO production associated with their viability and rapid increase of apoptosis. High resolution MRI can track these high inflammation, tumor angiogenesis regions in tumors. In conclusion, acute NO production can be a cause of hypoxia induced apoptosis and possibly MRI visible indicator of inflamed tumor viability.

Keywords: tumor, hypoxia, cytokine, apoptosis, TNF-alpha

1 INTRODUCTION

Nitric oxide (NO), is a free radical, acts as a natural anticoagulant, vasodilator, neurotransmitter, and mediator of immune system function and is released through the intermittant catalytic action of constitutive NO synthase (cNOS). The inflammatory response in the lungs is mediated by cytokines. The transcription of iNOS is of particular importance in the lungs including its regulation by three cytokines - tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (INF-γ). NO production by iNOS in response to TNF-α, IL-1β, and INF-γ has been reported as alveolar damage, hypoxia and reperfusion. The accumulation of fluid as a result of NO production is MRI visible in early stage of disease. It is controversial if NO production is defensive of consequence of hypoxia-induced apoptosis. We have recently reported a detailed investigation of the synergistic response of A549 cells (human alveolar epithelial cell line) to TNF-α, IL-1β, and INF-γ[1,2]. To understand the early events associated with MRI, we characterized role of cytokines on NO production and expression of iNOS in this report. The goal of our current study is to investigate the mechanism of synergy among TNF-α, IL-1β, or INF-γ. We proposed the hypotheses:

1) Cell exposure to combinations of cytokines results in larger nuclear concentrations of transcription factors that augment iNOS mRNA transcription and subsequent translation.
2) Cell exposure to any of the individual cytokines TNF-α, IL-1β, or INF-γ leads to iNOS monomer production, but only IL-1β and INF-γ exposure also leads to parallel biosynthesis of the cofactors necessary to form the functional iNOS dimer. We identified synergistic iNOS monomer production and the parallel biosynthesis of BH4 as key mechanisms underlying the synergistic response between IL-1β, INF-γ, and TNF-α in A549 cells.

2 MATERIALS AND METHODS

The following methods were used as described elsewhere[2].

- Cell culture and Exposure to Cytokines and Co-Factors
- Preparation of Cytosolic and Nuclear Extracts
- Western Blot Analysis
- Nitric Oxide Assay

Data Analysis: The concentration of NF-κB is:

$$F_{i,l} = \frac{C_i(t) - C_i(0)}{\left(C_i(t) - C_i(0)\right)_{i,max}} \quad (1)$$

where the subscripts "i" and "l" refer to the protein (iκB, NF-κB, or iNOS) and cytokine (IL-1β, INF-γ, and TNF-α), respectively, C is concentration (determined from the density of the Western Blot (see below), and the subscript "max" refers to the maximum value for protein "i".

By Western Blot, the response of the iNOS protein:

$$F_{iNOS,l} = \frac{\left(D_{iNOS}(t)\right)^{0.41} - \left(D_{iNOS}(0)\right)^{0.41}}{\left(\left(D_{iNOS}(t)\right)^{0.41} - \left(D_{iNOS}(0)\right)^{0.41}\right)_{iNOS,l,max}} \quad (2)$$

where the exponent of 0.41 was determined using non-linear least squares (R^2=0.97).

The synergy between the cytokines predicted values, $F^{P,II}$, as the sum of the response of the two individual cytokines present alone, and the synergistic effect, $S_{i,j}$ is the difference between the observed and predicted:

$$F_{i,lm}^{P,II} = F_{i,l} + F_{i,m} \quad (3)$$

$$S_{i,lm}^{II} = F_{i,lm} - F_{i,lm}^{P,II} \quad (4)$$

where the subscript "l" or "m" represent any two of the three cytokines. Three-way predicted values, $F^{P,III}$, are defined as the sum of the response of the individual and two-way synergy, and the synergistic effect, $S_{i,j}$ is the difference between the observed and predicted in the following fashion:

$$F_i^{P,III} = F_{i,l} + F_{i,m} + F_{i,n} + S_{i,lm}^{II} + S_{i,mn}^{II} + S_{i,ln}^{II} \quad (5)$$

$$S_i^{III} = F_i - F_i^{P,III} \quad (6)$$

3 RESULTS

3.1 Activation of NF-κB following Exposure of Cytokine(s)-

NF-κB exists in the cytosol as an inactive complex, NF-κB-IκB. Upon cytokine exposure, IκB is phosphorylated and rapidly degraded by intracellular proteolytic enzymes. As a result, NF-κB is activated and translocated into the nucleus. IκBα was analyzed by western blot in order to identify the activation of NF-κB in the cytosol of A549 cells. Both IL-1β (Figure 1A) and TNF-α (Figure 1B) cause a rapid fall in the levels of IκBα within 2 hours of exposure. The level of IκBα returns to normal within 4 hours post-exposure and remains unchanged through 24 hours. In contrast, IFN-γ did not cause a decrease in IκBα (Figure 1C). When A549 cells are exposed to both IL-1β and TNF-α in combination (Figure 1D) there is a synergistic (measured concentration different than predicted) decrease in IκBα within 2 hours, followed by a synergistic increase in IκBα at 4 hours to a higher level than predicted. This "rebound" or synergy effect is attenuated over the next 20 hours (Figure 1). Interestingly, IL-1β-induced IκBα degradation (or NF-κB activation) was augmented by IFN-γ (see Figure 5.1E at time = 2 hours) whereas TNF-α-induced IκBα degradation was not effected (Figure 1F). When all three cytokines are present (Figure 1G), there is a mild synergistic effect at 24 hours only.

3.2 NF-κB Translocation into the Nucleus- NF-κB is retained in the cytosol prior to cytokine exposure. NF-κB is a heterodimer -- p65/p50. The cytosolic levels of p65 do not change appreciably following exposure to IL-1β (Figure 2A and C, upper panels) whereas cytosolic p65 tends to increase following exposure to TNF-α (Figure 2B, upper panel) over 24 hours. In contrast, nuclear levels of NF-κB are increased dramatically above baseline within 2 hours of exposure to either TNF-α or IL-1β (Figure 2A and B, lower panels), but are unchanged following exposure to IFN-γ (Figure 2C, lower panel). When the cells were exposed to both IL-1β and TNF-α, the nuclear response is exaggerated (near additive), but is not synergistic (Figure 2D, lower panel). These results are consistent with Figure 1.

3.3 iNOS Expression following Exposure of Cytokine(s)-

All three cytokines induced mild iNOS monomer synthesis within 12 hours of exposure (Figure 1 A, B, and C). The levels of iNOS peak 24-48 hours following exposure. The interaction between IL-1β in combination with TNF-α and/or IFN-γ is strongly synergistic (Figure 1 D and E), particularly that between IL-1β and IFN-γ (~55% of maximal response); however, the synergy between TNF-α and IFN-γ (Figure 2F) is mild (increase from 2.5% to 5% of maximum). There may also exist a three-way synergy (Figure 1G) between IL-1β, TNF-α, and IFN-γ, particularly at 24 and 72 hours. This pattern of iNOS expression following exposure of cytokines is consistent with that seen for activation and translocation of NF-κB with the notable exception that IFN-γ can induce iNOS expression independently, yet does not activate NF-κB.

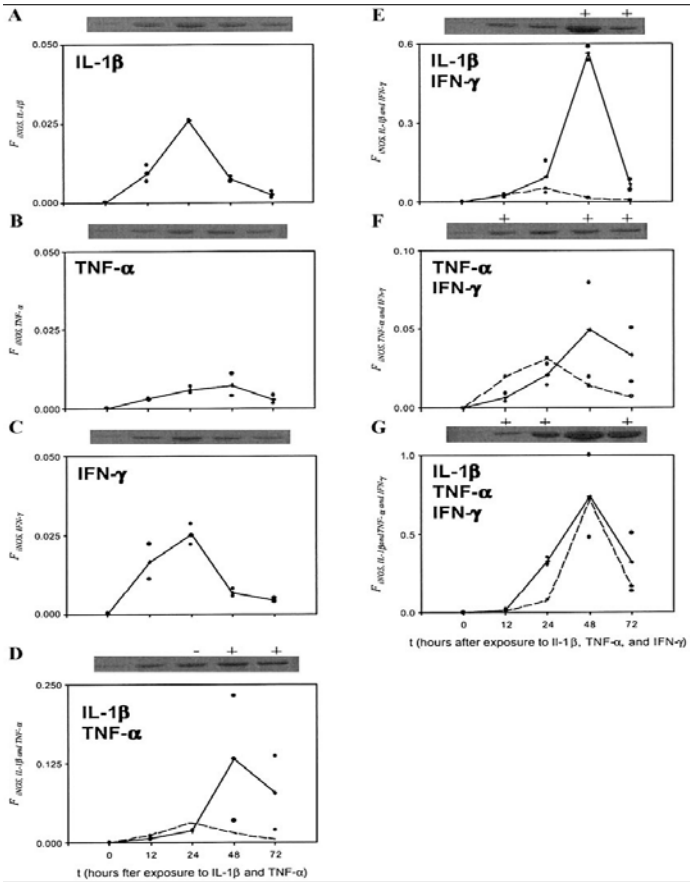

Figure 1:(On left) Expression of iNOS protein monomer in the presence of IL-1β(A). iNOS protein(A), TNF-α(B),IFN-α(C). IL-1β and TNF-α(D), IL-1β and IFN-γ(E) TNF-α and IFN-γ(F), IL-1β and TNF-α(G). The iNOS protein monomer was analyzed by western blot at 12, 24, 48, 72 hour interval. Two experimental values show each combination (•). Straight lines with small cross (+) represent average of two experiments (•). Dashed lines represent predicted values calculated by Eq 3.

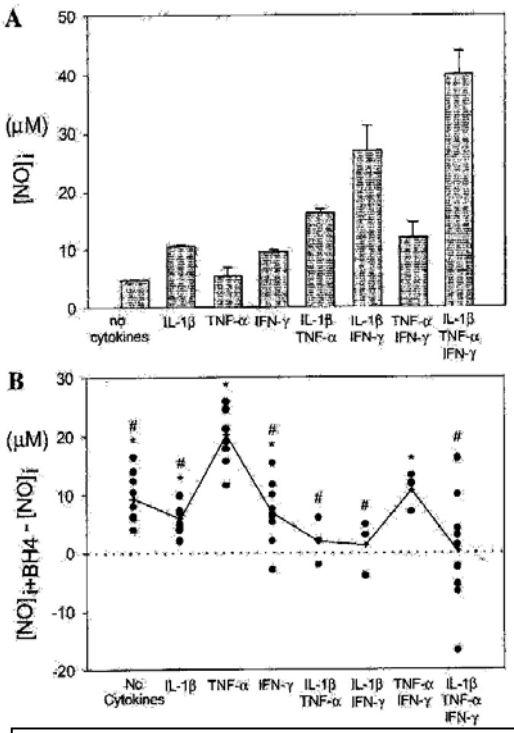

Figure 2: (On right) Effect of BH4 shows effect on NO production under various combinations of cytokines. NO was measured at 96 hours following exposure of cytokine(s). 0.1 μM of BH4 was added to the media.

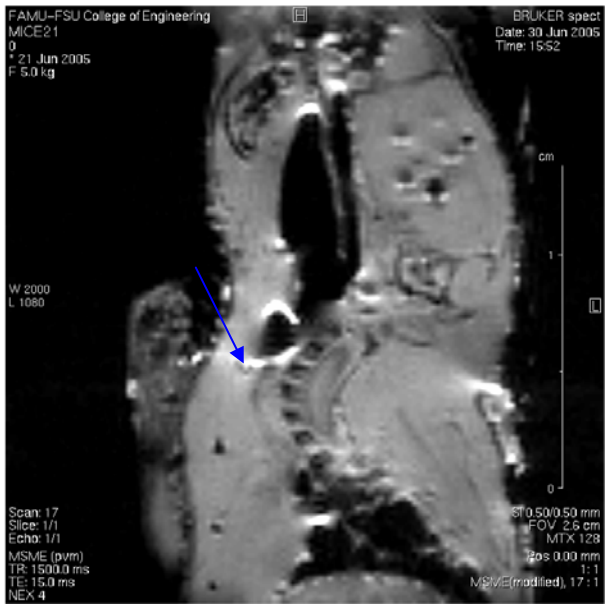

Figure 3: A mice lung is shown with site of inflammation as brighter on proton density MRI images. The images were acquired by Multislice-mutiecho PARAVISION sequence at TE15 ms, TR=1500 ms, matrix 128 x 128, using 500 MHz DRX bruker microimager. Note the enhanced brightmess on left side shown with arrow.

3.4 Effects of FAD, FMN, and L-Arginine on NO production-

Three cofactors (FAD, FMN, and BH4) and L-arginine modulated iNOS dimerization and its catalytic activity. All three cofactors affected NO production in the absence of cytokines. The FAD, FMN, BH4, and L-arginine, acted optimally at: 10 μM, 5 μM, 0.1 μM, and 2 μM. FAD, FMN, and L-Arginine did not affect *cytokine-induced* NO production. However, exogenous BH4 did impact *cytokine-induced* NO production (Figure 2), particularly TNF-α-induced NO production.

4 DISCUSSION

The study provided evidence to support a mechanism(s) of synergistic cytokine-induced NO production in human alveolar epithelial cells as early event of fluid accumulation in alveoli. The current report explores early molecular steps in the NO synthesis pathway as potential points of synergistic action they may help in interpretation of MRI visible area as brighter as shown in Figure 3.

iNOS Expression There was synergistic interaction between IL-1β in combination with IFN-γ and/or TNF-α on iNOS expression. In the presence of all three cytokines, there is not a convincing three-way synergy. Synergy between TNF-α and IFN-γ is subtle and cannot be ruled out, particularly 72 hours following exposure (note the difference in scale of Figure 2 E and F). This pattern of synergy is consistent with the discussion above regarding the presence of additional transcription factors. These findings provide strong evidence that the observed synergy in NO-production may be tightly linked to the amount of iNOS expressed. The interaction of IFN-γ with IL-1β or TNF-α may induce iNOS monomer synthesis.

Co-factors for iNOS Dimerization There are three co-factors necessary to produce an active iNOS dimmer and monomer: FMN, FAD, and BH4 in presence of Guanosine 5'-triphosphate (GTP) cyclohydrolase I, the rate limiting enzyme of BH4 biosynthesis, is induced in parallel to NOS induction [66, 68] (see Figure 2). NOS dimers have two heme groups that are essential for catalysis. BH4 stabilizes the dimeric, active state of the enzyme. Interestingly, NO, the final product, inhibits the binding of transcription factors to its cognate sequences on iNOS DNA, and can also attenuate the process of dimerization, conformational change, and activation, by forming a heme-NO complex. BH4 indirectly scavenges free NO by reaction with superoxide anion that is formed in the course of autooxidation of BH4. In addition, allosteric actions of BH4 on the iNOS domain destabilize heme-NO complex. Thus, newly synthesized iNOS monomers require an adequate supply of BH4 to become active, and to remain active.

Although TNF-α can induce the synthesis of iNOS monomers, it cannot induce NO production alone in A549. Interestingly, the presence of IFN-γ and TNF-α do not

produce NO synergistically. This suggests that in the presence of both IFN-γ and TNF-α, perhaps IFN-γ can induce cellular synthesis of BH4, but not in adequate amounts to capitalize on the additional iNOS monomers present from TNF-α.

5 CONCLUSION

There was synergy in NO production between IL-1□, IFN-□, and TNF-□ due to two mechanisms: 1) synergy in iNOS monomer synthesis, and 2) synergy in parallel BH4 synthesis. The increased level of iNOS synthesis is likely the result of non-linear interactions between several transcription factors including NF-□B and IRF-1. BH4 is an essential cofactor synthesized in parallel with the expression of inflammatory proteins regulated by cytokines during early stage of fluid accumulation appearing as MRI visible. It remains to understand if inflammatory induced NO production can be manipulated for therapeutic potential using MRI.