NFκB plays a role in basal levels of ROS in HCT116 cells

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Introduction
Colorectal cancer (CRC) is the third most common cancer worldwide and the disease rate is about 9% of the estimated cancer deaths in the US in 2015 [1]. Despite of advances in the therapy, survival rate (5 years) has not changed and chemotherapy still is the mainstay of treatment [2, 3]. In CRC therapy, fluoropyrimidines, in particular, 5-FU and its nucleoside analog 5′-fluoro-2′-deoxyuridine (FdUrd) are widely used agents [4, 5]. Investigating and understanding potential determinants of fluoropyrimidine metabolism could help to establish a strong rationale for improving therapeutic response.

Nuclear factor kappa B (NFκB) is a transcription factor that binds to specific sites on DNA and influences downstream expression of many genes involved in immune response, apoptosis, cell proliferation, adhesion and differentiation [6, 7]. Its function is inhibited by binding to the so-called Inhibitor of kappa-B-α (IxBα). Activation of NFκB follows the 26S proteosome-mediated degradation of IxBα that results from phosphorylation of IxBα by IkB kinase-β (IKKβ) [7]. NFκB signaling is a central coordinator of the innate and adaptive immune system. It is widely accepted that NFκB functions as an anti-apoptotic factor. Consistent with this, many investigations have shown that low levels of NFκB make cells vulnerable to the cytotoxic effects of thymidylate synthase (TS) inhibitors such as 5-FU, FdUrd [8-12].

In direct opposition to the generally accepted view, our laboratory has shown that NFκB promotes apoptosis during thymidylate deprivation, implicating a pro-apoptotic role in cells responding to TS inhibitors [13, 14]. Other studies have also shown a pro-apoptotic function for NFκB, implicating its dual role in the regulation of apoptosis [15, 16].

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The activation of NFκB is therefore a double-edged sword, and its function depends on context of signaling pathways.

To show that the apoptotic response to TS inhibitors is regulated by NFκB in human colon carcinoma cells, our lab utilized a NFκB deficient cell line, HCT116-dnIκBα, and its revertent, HCT116-dnIκBα-R [13]. HCT116-dnIκBα cell line is produced by stably transfecting HCT116 cells with a plasmid that expresses a dominant-negative mutant form of IκBα. To produce HCT116-dnIκBα-R cell line, HCT116-dnIκBα cells were passaged in non-selective media for 4 months and a revertent clone was obtained. It has been previously indicated that cell death mediated by TS inhibitors is ROS-dependent, and that NOX enzyme is involved [17]. Whether there is a link between ROS and transcription factor NFκB in apoptotic response to TS inhibitors is unknown. Here, we demonstrate that basal ROS levels were increased in HCT116-dnIκBα cells compared to HCT116 and HCT116-dnIκBα-R cells, however; the induced levels of ROS by TS inhibitors were not significantly changed in these cells. In addition to this finding, mRNA levels of p67phox were increased in HCT116-dnIκBα cells compared to HCT116 and HCT116-dnIκBα-R cells, indicating parallel changes with levels of ROS. We also demonstrate that drug-induced increases in NOX activity were similar in all three cells. Overall, these observations have led to the conclusion that NFκB acts on basal, but not drug-mediated ROS levels.

Material and Methods

Chemicals and Reagents
We obtained RPMI-1640 growth medium from Cellgro (Manassas, VA, USA), fetal bovine serum (FBS) from Atlanta Biologicals (Flowery Branch, GA, USA), cell permeable dyes, 2′,7′-dichlorodihydrofluorescin diacetate (H₂DCFDA) and dihydroethidium (DHE) from Invitrogen/Molecular Probes Inc. (Eugene, OR, USA), paraformaldehyde from Alfa Aesar (Ward Hill, MA, USA), permount medium, G418, phosphate saline buffer (PBS) and bovine serum albumin (BSA) from Fisher Scientific (Fair Lawn, New Jersey, USA), Power SYBR Green PCR Master Mix from Applied Biosystems (Foster City, CA), EGTA from Research Organics (Cleveland, OH, USA), sucrose from Mallinckrodt Chemical (Paris, KY, USA), NADPH from Calbiochem/EMD Biosciences (San Diego, CA, USA), lucigenin from Enzo Life Sciences Inc. (Plymouth Meeting, PA, USA), 5-FU and FdUrd from Sigma-Aldrich Co. (St. Louis, MO, USA). Cell culture chamber slides were obtained from VWR International LLC (Suwanee, GA, USA). Primers for p67phox were obtained from Integrated DNA Technologies Inc. (Coralville, IA, USA)

Cell culture
Human colon tumor cell line, HCT116 was kindly obtained from Dr. Michael G. Brattain. NFκB-deficient cell line, HCT116/dnIκBα, and its revertent cell line, HCT116/dnIκBα-R was previously described [13]. Cells were grown in RPMI-1640 medium supplemented with 10% heat-activated FBS at 37 ºC in a humidified 5% CO₂ atmosphere. Additionally, HCT116/dnIκBα cells were grown with 1mg/ml G418 to stabilize clone.

Fluorescence Microscopy
Intracellular ROS production was measured by oxidation of H₂DCFDA and DHE to fluorescent DCF by H₂O₂ and to fluorescent 2-hydroxyethidium (HE) by O₂⁻ respectively. Cells were grown to 60% confluence in chamber slides. After incubation, they were washed twice with PBS and were fixed in 4% paraformaldehyde for 30 min at room temperature. Cells were washed twice with PBS and separately stained with 5µM H₂DCFDA and DHE in dark followed incubation at 37°C for 30 min. Slides were kept in dark after this point. After incubation, cells were washed twice with PBS and chambers were separated from slides by slide separator. Coverslips were mounted on glass slides using permount medium. Images were acquired at room temperature using an inverted microscope (Axiovert 200, Carl Zeiss) equipped with a Plan-Apo 63×/1.40 objective and a charge-coupled camera (AxioCam HRm, Carl Zeiss) linked to AxioVision software (version 4.7, Carl Zeiss). To get the best visual reproduction, the image input levels were adjusted by stretching the histogram. In this point, image brightness and linearity were sustained. To analyze changes in DCF and HE fluorescence, ImageJ software (National Institutes of Health) was used to get fluorescence in numbers. Analysis of DCF and HE fluorescence was done by selecting cells with a pixel range for brightness and by subtracting background stained.
Flow Cytometry
Cells were grown to 80% confluence in 60 mm plates. After incubation, they were washed twice and suspended with fluorescence-activated cell-sorting (FACS) staining buffer (PBS with 1% BSA). 0.5 μM H$_2$DCFDA and DHE dyes were separately applied to cells for at 37 °C for 15min in dark. After this point, cells were kept in dark. They were washed twice, suspended with FACS buffer and analyzed by flow cytometer (FC 500 by Beckman Coulter Fort Collins Co). Histograms were analyzed in CXP software by obtaining X-mean value for each treatment. From 3 separate experiments, average X-mean was calculated for each treatment and data was normalized in fold increase of drug treatments to control.

Quantitative PCR
According to the manufacturer's suggestions, cDNA (1 μl) was amplified using Power SYBR Green PCR Master Mix. Reactions were run for one cycle at 95 °C for 10 min, and 40 cycles of 95 °C for 15 s, 50 °C for 10 s and 72 °C for 40 s using an Applied Biosystems 7300 Real Time PCR System. All mRNA levels were quantified relative to GAPDH by measuring SYBR green incorporation during quantitative PCR using the relative standard curve method. Statistical analyses and calculations were done as described in the manufacturer's protocol. Relative changes in each gene levels between drug-treated and nontreated cells were expressed as fold induction compared with the basal level of expression in nontreated cells. Primers used for p67phox were; sense 5' ACC AGA AGC ATT AAC CGA GAC3', antisense 5'TTC CCT CGA AGC TGA ATC AAG3', and for GAPDH, sense 5'TCC CTG AGC TGA ACG GGA AG3', antisense 5'GGA GGA GTG GGT GTC GCT GT3'.

NADPH Oxidase Assay
Cells were washed twice with PBS after incubation. 10⁵ cells for each cell line and treatment were suspended in 500 μl reaction buffer [50 mM phosphate buffer (pH 7.0), 1 mM EGTA, 150 mM sucrose]. 100 μM NADPH was added as substrate. Light sensitive 5 μM lucigenin was added to reaction mixture in dark. Cells were kept in dark after this point and incubated at 37 °C for 10 min. Negative controls like adding only NADPH to the reaction mixture without cells and adding only cells to the mixture without NADPH were prepared. After incubation, NADPH oxidase activity was immediately detected by lucigenin-derived chemiluminescence using a luminometer (GloMax-20/20 luminometer, Promega, Madison, WI, USA). The levels of emitted chemiluminescence in every 15 s for 1 min of measurement were saved for each treatment. The signal reading was normalized by subtracting chemiluminescence obtained from negative controls and expressed as arbitrary light units per 10⁶ cells.

Statistical Analysis
Experiments were independently repeated at least three times. Figures are representative of all the corresponding experiments performed. Results are presented as the mean ± SEM and a paired two-sided Student’s t test was used to calculate statistical significance. Differences with p values of 0.05 or less were considered statistically significant.

Results
ROS formation mediated by TS inhibitors is not NFκB-dependent
NFκB regulates the expression of many genes for immune response, cell proliferation and differentiation, etc. Imbalance between NFκB and its inhibitor IκB has been associated with progress of many diseases, including cancer [7]. It is suggested that NFκB plays a pro-apoptotic role in apoptotic response to TS inhibitors [13, 14]. To determine whether it acts on oxidative or ROS-derived apoptotic response to TS inhibitors, we exposed parental line HCT116, NFκB deficient cell line, HCT116-dnIkBα, and its revertent, HCT116-dnIkBα-R to TS inhibitors. Basal H$_2$O$_2$ and O$_2^−$ levels were higher in HCT116-dnIkBα cells as compared to HCT116, and were rescued in HCT116-dnIkBα-R cells. However, both H$_2$O$_2$ and O$_2^−$ levels induced by TS inhibitors were unchanged in all three lines (Figure 1).

Loss of NFκB does not alter mRNA levels of p67phox induced by FdUrd
As previously reported, p67phox induction is associated with in drug-mediated ROS generation in HCT116 cells [17]. In order to determine if NFκB plays a role in the induction of p67phox mRNA induced by TS inhibitors, we treated HCT116, HCT116-dnIkBα, and HCT116-dnIkBα-R cells with inhibitors and found that loss of NFκB increased basal levels of p67phox mRNA in HCT116-dnIkBα relative to HCT116 and HCT116-dnIkBα-R cells. However, NFκB deficiency did not significantly change mRNA levels of p67phox in drug-treated cells (Figure 2).
NFκB deficiency does not affect increases in NOX activity mediated by TS inhibitors

In order to examine whether drug-mediated increases in NOX activity are altered by NFκB, we exposed HCT116, HCT116-dnIkBα, and HCT116-dnIkBα-R cells to TS inhibitors and measured NOX activity. We determined that loss of NFκB did not alter increases in NOX activity mediated by TS inhibitors in any of the three cells (Figure 3).

Discussion

NFκB is a multipotent transcription factor that regulates cell proliferation and differentiation, immune response, apoptosis and other integral cellular functions [6, 7]. Although several studies have reported it as having a pro-apoptotic role [13, 14], most investigations have indicated it to be an anti-apoptotic factor [8, 9, 18], showing its dual functions in the control of cell functions. We have found that NFκB deficiency increases basal levels of ROS and p67phox mRNA. However, it has no effect on drug-mediated ROS production and p67phox expression.

It is known that NFκB promotes cell death due to thymidylate deprivation resulting from TS inhibition. However, the mechanism underlying NFκB-mediated cell death is not well understood. Our data suggests that this does not occur via alterations in ROS induction mediated by TS inhibitors. We have shown that HCT116-dnIkBα, NFκB deficient, cells had high basal levels of ROS compared to HCT116 and HCT116-dnIkBα-R cells. This might suggest that absence of NFκB results in abnormalities in cells because it affects expressions of many genes in cellular metabolism, giving rise to oxidative stress. Due to loss of NFκB, TS inhibitors do not further induce ROS generation in HCT116-dnIkBα cells compared to HCT116 and HCT116-dnIkBα-R cells.

Figure 1. Loss of NFκB increases basal ROS levels. HCT116, HCT116/dnIkBα and HCT116/dnIkBα-R cells were treated with 10 µM 5-FU, and 10 µM FdUrd for 24 h and then stained by 5 µM H2DCF-DA and 5 µM DHE. ROS detection was done in both fluorescence microscopy and flow cytometry. Pictures from fluorescence microscopy represent H2O2 formation (green) and O2·− (red). Bars from flow cytometry represent fold increase of H2O2 (DCF fluorescence) and O2·− (HE fluorescence) formation ± SEM from 3 experiments (UT: untreated, 5-FU: fluorouracil, FdUrd: 5′-fluoro-2′-deoxyuridine; * p<0.05, ** p<0.01).
cells, indicating the effect of NFκB on drug-mediated ROS generation.

We have also shown that basal levels of p67phox mRNA were increased in HCT116-dnIkBα cells relative to HCT116 and HCT116-dnIkBα-R cells, but induced mRNA levels by TS inhibitors is similar between these 3 cells. Additionally, increases in NOX activity are also similar in these cells, indicating that under the conditions of our experiments, NFκB has no role in NOX activity in response to TS-directed agents.

Under certain conditions, it is suggested that pro-apoptotic role of NFκB in thymidylate deficiency might be ROS-dependent, but it is not due to p67phox or NOX enzyme. It will be important to reveal the mechanism by which it promotes apoptosis in future work and to determine how the anti-apoptotic responses were silenced.

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REFERENCES

NFκB and oxidative stress


