Regulation of c-FLIP promoter activity by superoxide anions in cancer cells

Krishna S., Subramaniam K. and Pervaiz S.

Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Blk MD9, 2 Medical Drive Singapore 117597

ABSTRACT

One of the key contributors to tumorigenesis in cancer cells is evasion of apoptosis. c-FLIP, an inhibitor of the death receptor pathway of apoptosis is overexpressed in many cancers. Reactive oxygen species (ROS) play an important part in regulating many transcriptional regulators of c-FLIP. As a slight increase in the intracellular superoxide levels can inhibit apoptosis, our aim was to analyze the transcriptional regulation of c-FLIP by superoxide production. Changes in c-FLIP full length promoter and 5’ deletion constructs induced by superoxide were observed to determine the region of the promoter affected. Our results indicate a superoxide mediated increase in c-FLIP promoter activity and promoter analysis revealed Egr-1, E2F and AP-1 as important transcription factor binding sites. These data will provide greater insight into the redox regulation of c-FLIP transcription.

INTRODUCTION

Cancer is a class of diseases which involves dynamic changes in the genome and is characterized by uncontrolled cell proliferation. Resistance towards apoptosis by deregulation of proteins involved in controlling the mitochondrial or the death receptor pathway of apoptosis is a major contributor to this transformation.

Cellular FLICE-like inhibitory protein (c-FLIP) inhibits the death receptor pathway by inhibiting the activation of caspase-8 and is upregulated or overexpressed in many cancers. Its regulation at transcriptional level is modulated by important transcription factors like NF-κB, AP-1, E2F and FOXO3a (Li et al., 2007). Reactive oxygen species (ROS) at lower concentrations function as secondary messengers and modulate the activity of some of the c-FLIP transcription factors. A slight increase in the intracellular superoxide levels can inhibit apoptosis, whereas a slight increase in H₂O₂ favours apoptosis and tumor cells characteristically exhibit higher superoxide levels (Clement and Pervaiz, 1999).

On the basis of these previous reports, it could be hypothesized that increased superoxide levels could directly increase c-FLIP expression by altering the activity or expression of a transcription factor(s) that regulates c-FLIP, which could also serve as a model for the pro-oxidant state in tumors. The aim of the present study was to observe whether c-FLIP expression is increased by superoxide and to identify the possible transcription factors that may be involved in mediating this increase. We observed the changes in c-FLIP promoter activity induced by superoxide, used 5’ deletions to determine the region of the promoter that may be affected and then analyzed this region for putative transcription factor binding sites.
MATERIALS AND METHODS

Plasmids and DNA purification
A 1460bp sequence containing the promoter region of c-FLIP (L) (–1179 to +281bp, Accession Number AB038965) cloned into luciferase-expressing reporter plasmid pGL3-basic was kindly provided as a gift by Dr. Wafik El-Deiry (University of Pennsylvania School of Medicine, USA). pRL-TK (renilla-luciferase expressing plasmid) and the three 5’ deletions – Deletion-1 (-741 to +281bp), Deletion-2 (-303 to +281bp) and Deletion-3 (-11 to +281bp) had been made previously in the lab. Plasmids were purified using the Nucleobond Xtra Kit (Machery-Nagel, Germany).

Flow cytometric measurement of intracellular superoxide
Superoxide production was quantified using dihydroethidium. HeLa cells were incubated with 50µM, 100µM and 200µM DDC for 2 and 4 hrs each. Cells were stained with 2.5µM HE for 20 minutes. At least 20,000 events were analyzed by flow cytometry (Coulter EPICS Altra; Beckman Coulter, Fullerton, CA). The data obtained was analyzed with WinMDI software.

Transient Transfections, Luciferase Assays and Sequence Analysis
The full length c-FLIP promoter and all the deletion constructs pGL3 (5µg/well) and pRL-TK (100ng/well; Renilla luciferase for normalization) were transfected in HeLa cells using modified calcium phosphate method as previously described (Niehof et al., 1995). After 24 hours of incubation, cells were treated with or without 50µM DDC for 4hrs. The cells lysed with reporter lysis buffer (Promega, WI, USA) and luciferase activity was assayed using Dual Glo Luciferase assay system (Promega, WI, USA). The light units of luciferase activity were normalized with renilla to control for transfection efficiency. Results are shown as a % increase in activity relative to untreated cells. For the prediction of possible binding sites for transcription factors, Alibaba 2.1 and P-match softwares were used.

RESULTS

Our results show an increase in intracellular superoxide levels with 50µM DDC for 4hrs (Fig 1A). This result correlates with c-FLIP promoter activity as HeLa cells when transfected with the full length c-FLIP promoter and exposed to 50µM DDC for 4 hours showed a 40% increase in luciferase activity (Fig 1B).

Cells transfected with the full length promoter or the three 5’ deletion constructs when exposed to 50µM DDC for 4 hours, show a 40% and 56% increase in the full length and Deletion-1 respectively while Deletion-2 and Deletion-3 showed no increase (Fig. 1C) indicating that Deletion-1 (-741 to +281bp) may contain the region that is being affected by increased superoxide levels in the cell.

Sequence analysis of Deletion-1 (-741 to +281bp) revealed AP-1 (-713 to -704bp), E2F (-559 to -552bp) and Egr-1 (two sites in this region, at -729 to -720bp and -334 to -324bp) as putative transcription factor binding sites that may be important in c-FLIP transcriptional regulation and are uniquely found in this region.
DISCUSSION

Our results support the hypothesis that intracellular superoxide production could be causing an increase in c-FLIP promoter activity. Previous studies have indicated that superoxide anion is a natural inhibitor of death receptor signaling (Clement and Stamenkovic, 1996). We provide a mechanistic link indicating that superoxide could be inhibiting Fas-mediated cell death by increasing the expression of c-FLIP. This model is of particular importance in tumor cells since they characteristically exhibit higher superoxide concentrations and this provides them with a growth advantage due to a resistance to apoptosis (Cerutti, 1985). This pro-oxidant state in tumor cells may be causing c-FLIP overexpression thereby inhibiting death receptor mediated apoptosis.

AP-1, Egr-1 and E2F are all downstream effectors of the MAPK pathway which regulates cell division, apoptosis and cell differentiation by activation of kinases ERK, JNK and p38 (Zhou et al., 2006). Egr-1 and E2F are both activated indirectly by ERK. ERK activates an important transcription factor CREB which in turn increases the expression of as Egr-1 (Murphy et al., 2005). Similarly, ERK activates c-myc transcription factor which upregulates expression of E2F (Murphy et al., 2005).
et al., 2005). Also, it has been reported that ERK is activated by superoxide anions (Navaro et al., 2006). Taking this into consideration with our findings, it can be hypothesized that superoxide could be causing ERK activation, which could in turn activate c-myc and CREB and their downstream targets E2F and Egr21 respectively.

AP-1 is mainly activated by its N-terminal phosphorylation by JNK (Eferl and Wagner, 2003). The regulation of JNK in tumor cells by a mild increase in ROS has not been established, though JNK is shown to be activated by \( \text{H}_2\text{O}_2 \) (Zhou et al., 2006). However, since a tight balance between \( \text{H}_2\text{O}_2 \) and superoxide usually determines the effector function, it could be assumed that by inhibiting Cu/Zn SOD, the \( \text{H}_2\text{O}_2 \) levels go down and thus reduced activation of JNK could occur in the presence of an environment with increased superoxide. Taking this into consideration with our findings, increased superoxide could cause reduced activation of JNK, which in turn could reduce AP-1 activation. Reduced AP-1 activation would cause increase in c-FLIP promoter activity.

Unifying these pathways with the pro-oxidant tumor model that we described, it could be concluded that the higher concentration of superoxide anions in tumors could cause activation of ERK and possible inhibition of JNK. Both could cause increased c-FLIP expression and thus promote tumor growth. Thus c-FLIP could possibly be an integral part of ERK-JNK-MAPK signaling which could be the missing link between superoxide production and c-FLIP transcription in our model. On the whole, this study can serve as a basis for understanding the biology of cancer cells in relation to the intracellular redox state. Further characterization of the superoxide mediated regulation of ERK and JNK will uncover the mechanisms by which superoxide inhibits apoptosis in cancer cells.

REFERENCES