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Different roles of ROS and Nrf2 in Cr(VI)-induced inflammatory responses in normal and Cr(VI)-transformed cells

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Abstract

Hexavalent chromium (Cr(VI)) is classified as a human carcinogen. Cr(VI) has been associated with adenocarcinomas and squamous cell carcinoma of the lung. The present study shows that acute Cr(VI) treatment in human bronchial epithelial cells (BEAS-2B) increased inflammatory responses (TNF- α , COX-2, and NF- κ B/p65) and expression of Nrf2. Cr(VI)-induced generation of reactive oxygen species (ROS) are responsible for increased inflammation. Despite the fact that Nrf2 is a master regulator of response to oxidative stress, silencing of Nrf2 in the acute Cr(VI) treatment had no effect on Cr(VI)-induced inflammation. In contrast, in Cr(VI)-transformed (CrT) cells, Nrf2 is constitutively activated. Knock-down of this protein resulted in decreased inflammation, while silencing of SOD2 and CAT had no effect in the expression of these inflammatory proteins. Results obtained from the knock-down of Nrf2 in CrT cells are very different from the results obtained in the acute Cr(VI) treatment. In BEAS-2B cells, knock-down of Nrf2 had no effect in the inflammation levels, while in CrT cells a decrease in the expression of inflammation markers was observed. These results indicate that before transformation, ROS plays a critical role while Nrf2 not in Cr(VI)-induced inflammation, whereas after transformation (CrT cells), Nrf2 is constitutively activated and this protein maintains inflammation while ROS not. Constitutively high levels of Nrf2 in CrT binds to the promoter regions of COX-2 and TNF- α , leading to increased inflammation. Collectively, our results demonstrate that before cell transformation ROS are important in Cr(VI)-induced inflammation and after transformation a constitutively high level of Nrf2 is important.

Keywords

Hexavalent chromium; Inflammation; Transformed cells; ROS; Nrf2

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1. Introduction

Hexavalent chromium Cr(VI) is classified as a human carcinogen. Exposure to Cr(VI) occurs in multiple occupational environments due to its commercial usage in welding, chrome pigmenting, leather tanning, and ferrochrome industry (Fishbein, 1981; Malsch et al., 1994). Cr(VI) is also a component of industrial waste and atmospheric pollution. Clinical studies link Cr(VI) exposure to high incidence of respiratory cancer (Gibb et al., 2000). Cr(VI) from natural and anthropogenic sources is present in drinking water as trivalent Cr(III) and hexavalent Cr(VI) forms (Ellis et al., 2002). When ingested, Cr(VI) is reduced to Cr(III) by saliva and gastric fluids (DeFlora et al., 1997; Proctor et al., 2012). Intra-cellular reduction of Cr(VI) to Cr(III) generates ROS that can damage proteins, lipids, and DNA (Hamilton and Wetterhahn, 1989; Stout et al., 2009). In addition, Cr(VI) interacts with DNA, resulting in development of intestinal tumors in mice (Lawrence et al., 2005; Thompson et al., 2014). National Toxicology Program (NTP) has reported that exposure of Cr(VI) in B6C3F1 mice, at doses higher than 20 mg/ml in drinking water, develops adenomas and carcinomas of duodenum and jejunum (Stout et al., 2009; O'Brien et al., 2013; Thompson et al., 2014).

Persistent inflammation contributes to cancer development (Coussens and Werb, 2001; Aggarwal et al., 2009). ROS generated from inflammatory cells or mitochondria act as the central endogenous carcinogen that drives cancer-promoting signaling pathways (Pratheeshkumar et al., 2014; Divya et al., 2015). After activation of cancer-promoting signaling pathway, inflammatory cells release cytokine -TNF- α that can promote chronic oxidative stress in the affected tissues (Mantovani et al., 2008; Colotta et al., 2009; Dennis et al., 2009; Mantovani et al., 2010). NADPH oxidase (NOX) is one of the major sources of cellular ROS. A previous study from our laboratory has shown the importance of NOX in Cr(VI)-induced ROS generation and carcinogenesis (Wang et al., 2011). In addition, Cr(VI) exposure also leads to activation of mitogen-activated protein kinases (MAPKs), including c-Jun. N-terminal kinase (JNK)1/2, p38 and extracellular-signal regulated kinase (ERK)1/2. Cr(VI) also induces inflammation response by activating crosstalk between NF- κ B and AP-1, resulting in induction of COX-2 (Zuo et al., 2012). It has been reported that in the lung tissue a single exposure to Cr(VI) induces toxic mucosal injury and acute inflammatory response, and upregulates IL-6, pAKT, and activates MMPs (Beaver et al., 2009). Azoxymethane/dextran sodium sulfate (AOM/DSS) pretreatment followed by Cr(VI) exposure has promotional effect on colorectal tumor incidence, multiplicity, size and grade as well as cell inflammatory response (Wang et al., 2012). Increased tumor incidence was accompanied with increase in β -catenin and phospho-GSK levels. This study indicates that carcinogenicity of Cr(VI) is mediated through ROS-Wnt/ β -catenin signaling pathway (Wang et al., 2012).

The transcription factor Nrf2, a basic region-leucine zipper transcription factor (Urano and Motohashi, 2011; Boddupalli et al., 2012), is the guardian of redox homeostasis. Under oxidative stress conditions, it activates a series of antioxidant and cytoprotective genes that share in common a *cis*-acting enhancer sequence, termed as antioxidant response element (ARE) (Hayes and McMahon, 2009; Saw et al., 2014). These protective enzymes are involved in detoxification of ROS and xenobiotic compounds (Itoh et al., 1997). Low levels

of Nrf2, or loss of Nrf2, increases ROS production and predisposes cells to tumorigenic state, whereas constitutively high levels of Nrf2 favor cancer cell survival against chemotherapeutic agents (Sykietis and Bohmann, 2008; Tullet et al., 2008). Two independent mechanisms for Nrf2 activation has been proposed: (a) dissociation of Nrf2 from Keap1 and (b) activation of protein kinases, such as PKC, results in phosphorylation of Nrf2 which increases stability of Nrf2, subsequently releases of Nrf2 from Keap1 (Huang et al., 2002; Wakabayashi et al., 2004; Osburn and Kensler, 2008; Klaunig et al., 2010).

Previous reports from our laboratory have shown that cadmium transformed cells exhibit higher apoptosis resistance as compared to normal BEAS-2B. In these transformed cells, Nrf2 is constitutively expressed. Silencing of Nrf2 increases apoptosis (Son et al., 2014). The present study explores the regulatory link between Cr(VI)-induced inflammatory responses and Nrf2. Herein, we provide evidence suggesting mechanistic differences in Cr(VI)-induced inflammatory responses between ROS and Nrf2 before and after Cr(VI)-induced transformation. Before transformation, ROS is important in Cr(VI)-induced inflammation, while after transformation a constitutively higher levels of Nrf2 is important.

2. Materials and methods

2.1. Antibodies and chemicals

Potassium dichromate ($K_2Cr_2O_7$), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) were purchased from Sigma-Aldrich (St Louis, MO). Dichlorodihydrofluorescein acetate (DCFDA) and dihydroethidium (DHE) were obtained from Molecular Probes (Eugene, OR). Lipofectamine 2000 was purchased from Invitrogen Corporation (Carlsbad, CA). Antibodies specific for NF- κ B/p65, COX-2, TNF- α , Nrf2, Lamin A were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against pNF- κ B/p65, I κ B α and pI κ B α were purchased from Cell Signaling Technology (Danvers, MA). Monoclonal anti-actin antibody produced in mice was from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture and reagents

BEAS-2B cells were purchased from American Type Culture Collection and Chromium transformed cells (CrT) cells were maintained in DMEM high glucose (Kim et al., 2015). All culture media were supplemented with 10% FBS and penicillin-streptomycin (Sigma). Cells were cultured at 37 °C under a humidified 95%:5 (v/v) mixtures of air and CO₂ (Roy et al., 2013).

2.3. Plasmids, siRNAs and transfection

The overexpression of catalase, SOD2 in BEAS-2B cells has been described previously (Son et al., 2014). Nrf2, SOD2 and catalase shRNAs were purchased from Origene (Rockville, MD) (Son et al., 2014; Son et al., 2015a). Transfections were performed using Lipofectamine TM 2000 (Invitrogen) according to the manufacturer's protocol.

2.4. Western blot and immunofluorescence

Western blotting was performed as described previously (Son et al., 2010b). Equal amounts of protein (30 μ g/sample) were separated by NuPAGE Bis-Tris electrophoresis system

(Invitrogen, Carlsbad, CA) and blotted onto nitrocellulose membrane (Whatman, Dassel, Germany). Blots were probed with primary and then secondary antibodies before exposure to Hyperfilm (Amersham Pharmacia Biotech).

For immunofluorescence analyses, cells were grown on glass cover-slips and treated with Cr(VI) as indicated. Cells were fixed in 4% paraformaldehyde, permeabilized using 0.1% TritonX-100 and incubated with primary antibody at room temperature. Secondary antibody Alexa Fluor 488 (Invitrogen) and Phalloidin 568 (Invitrogen), and nuclei were counterstained (Vector laboratories, H-1200). Images were acquired using Olympus, BX53.

To generate fractions enriched in nuclear and cytoplasmic proteins, 5×10^6 cells were harvested and washed in PBS. Next, the cells were resuspended in the lysis buffer (10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl at 7.5 pH, 0.1 mM PMSF, 1 mM DTT) and were pass through 25G needle 10 times using 1 ml syringe. After this, lysates were left on ice for 20 mins followed by centrifugation at 3000 rpm for 5 min. (nuclear). The nuclear pellets were re-suspended in lysis buffer and then sonicated. The remaining leftover supernatant was considered as cytoplasmic fraction.

2.5. Intracellular ROS determination

Cells were washed once with PBS and incubated with DCFDA or DHE (10 μ M) for 30 mins. Cells were harvested with trypsin, washed twice with cold PBS, and analyzed by fluorescence-activated cell sorting (FACS Calibur, BD Biosciences). The fluorescence intensity of DCFDA was measured at an excitation wavelength of 492 nm and an emission wave length of 517 nm. The fluorescence intensity of DHE was measured at an excitation wavelength of 535 nm and an emission wavelength of 610 nm. All measurements of ESR were conducted using Bruker EMX spectrometer (Bruker Instruments) and a flat cell assembly as described previously (Son et al., 2010a).

2.6. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a Pierce™ Agarose ChIP Kit (Thermo Scientific, Rockford, IL). Sheared chromatin was diluted and immunoprecipitated with 2 μ g of antibodies of Nrf2 or control IgG. DNA protein complexes were eluted from the protein A/G agarose beads using a spin column and were reverse cross-linked by incubating with NaCl at 65 °C. The binding of Nrf2 to the ARE regions of the TNF- α , and COX-2 (Gjyshi et al., 2014) was analyzed by PCR (Eppendorf, Foster City, CA).

2.7. Statistical analysis

Presented values are means $\pm$ SD. One-way analysis of variance (ANOVA) was used for statistical analysis, with $p < 0.05$ was considered significantly different.

3. Results

3.1. Acute Cr(VI) treatment induces Nrf2 and inflammatory responses in BE-AS-2B cells

To investigate the role of Cr(VI) in inducing inflammation, BEAS-2B cells were treated with different concentrations (1, 2.5 and 5 μ M) of Cr(VI) for 24 h and analyzed for expression of

Nrf2 and inflammatory markers-COX-2, TNF- α and NF- κ B. As expected, increased expressions of Nrf2, COX-2 and TNF- α were observed (Fig. 1A). Similarly, increased immunostaining intensities of both COX-2 and TNF- α (Fig. 1B–C) were also observed.

Cr(VI) is able to induce inflammatory responses by activating crosstalk between NF- κ B and AP-1, resulting in the induction of COX-2 (Zuo et al., 2012). To determine whether NF- κ B is involved in Cr(VI)-induced inflammation, expression level of NF- κ B family proteins were analyzed. Fig. 1D, shows that Cr(VI)-induced phosphorylation of I κ B α occurs in a dose dependent manner. Phosphorylation of I κ B α is required for NF- κ B activation (Lawrence et al., 2005). In correlation with the elevated phosphorylation of I κ B α , activation and translocation of p65 to the nucleus was observed (Fig. 1D–G). In summary, increase in Nrf2, as well as TNF α and COX-2, and nuclear localization of NF- κ B/p65 was triggered by the acute Cr(VI) treatment.

3.2. Short term Cr(VI) exposure induces ROS generation

Studies have shown that ROS induces inflammation and that ROS derived from inflammatory cells acts as carcinogen (Ye et al., 1999; Coussens and Werb, 2001; Aggarwal et al., 2009; Son et al., 2010a; Pratheeshkumar et al., 2014). To study the relationship between Cr(VI)-induced ROS generation and inflammation, ROS production was quantified by flow cytometry using the fluorescent probes DHE and DCFDA. Cr(VI) exposure dramatically stimulated H₂O₂ and O₂^{•−} generation in BEAS-2B cells, as indicated by an increase of DCFDA and DHE intensity, respectively in fluorescence microscopy (Fig. 2A). Similarly, Cr(VI)-induced H₂O₂ production was also detected by flow cytometry using DCFDA (Fig. 2B–C). Exogenous treatment of catalase significantly inhibited the Cr(VI)-induced ROS generation (Fig. 2B–C). Further, the Cr(VI)-induced •OH generation in BEAS-2B cells was detected by electron spin resonance (ESR). Fig. 2D shows that cells alone did not generate any detectable ESR signal, and that addition of Cr(VI) resulted in generation of a 1:2:2:1 quartet ESR signal, an indication of OH generation (Shi et al., 1999). These results indicate that Cr(VI) exposure induces ROS production in BEAS-2B cells.

3.3. Intracellular ROS production is essential for Cr(VI)-induced inflammatory responses in BEAS-2B cells

ROS have been regarded as a key mediator for Cr(VI)-induced inflammation (Coussens and Werb, 2001; Aggarwal et al., 2009; Pratheeshkumar et al., 2014). In a cellular system, scavenging or detoxification of ROS is mainly achieved by antioxidant enzymes: CAT and SOD. To investigate the role of ROS in mediating Cr(VI)-induced inflammation, BEAS-2B cells were transiently transfected with plasmid overexpressing SOD2 and CAT respectively.

Overexpression of SOD2 (Fig. 3A) and CAT (Fig. 3B) in BEAS-2B cells was confirmed by Western blotting. BEAS-2B cells overexpressing SOD2 and CAT were treated in presence or absence of Cr(VI) for the analysis of Nrf2 and inflammatory markers-COX-2, TNF- α and NF- κ B/p65. Overexpression of SOD2 and CAT in BEAS-2B suppressed the Cr(VI)-induced expression of Nrf2 and inflammatory markers -COX-2, TNF- α and NF- κ B/p65 (Fig. 3C–F). Compared to SOD2 higher inhibition of inflammatory markers were

observed in CAT overexpressed BEAS-2B cells. These results indicate that ROS production is essential for Cr(VI)-induced inflammation.

3.4. Nrf2 is not involved in Cr(VI)-induced inflammatory responses in BEAS-2B cells

High cellular concentration of ROS selectively activates-Nrf2, leading to increased binding of Nrf2 to the AREs in gene promoters, resulting in the activation of a broad spectrum of protective enzymes, including those involved in xenobiotic detoxification and antioxidative response (Moi et al., 1994; Itoh et al., 1997). To examine the contributions of Nrf2 in Cr(VI)-induced inflammation, BEAS-2B cells were transiently silenced with Nrf2 shRNA then exposed to Cr(VI). As shown in Fig. 4A, silencing of Nrf2 showed no noticeable changes in the inflammation levels. These results indicate that Cr(VI)-induced inflammation is independent of Nrf2 in BEAS-2B cells. Similarly, the levels of translocated NF- κ B/p65 remain unchanged in Nrf2 knockdown even after Cr(VI) treatment (Fig. 4B).

3.5. Cr(VI)-transformed cells exhibit high levels of inflammatory proteins and low levels of ROS

The above findings (Figs. 3-4) suggest that ROS is essential for Cr(VI)-induced inflammatory responses in BEAS-2B cells. To investigate whether this is also the case in CrT cells, we compared the basal levels of ROS, Nrf2, COX-2, TNF- α , NF- κ B/p65, CAT and SOD2 in the passage matched BEAS-2B and CrT cells. Higher basal levels of Nrf2 and inflammatory markers-COX-2, TNF- α and NF- κ B along with antioxidant enzymes were observed in CrT cells (Fig. 5A–B). In-contrast, when ROS levels were quantified by fluorescence produced by DCFDA, lower fluorescence intensity was observed in CrT cells (Fig. 5C–D). In summary, these results indicate elevated levels of antioxidant and inflammation accompanied by lower levels of ROS in CrT cells.

3.6. Nrf2 is essential for maintaining higher inflammation in CrT cells while ROS are not

Results in Fig. 3 show that ROS are essential for Cr(VI)-induced inflammatory responses in BEAS-2B cells. To investigate whether this is also the case in CrT cells, knock-down of highly expressed antioxidant enzymes-SOD2 and CAT were performed. Silencing of either SOD2 (Fig. 6A) or CAT (Fig. 6B) has no effect in the expression of Nrf2 and inflammatory markers-COX-2, TNF- α and NF- κ B in the CrT cells (Fig. 6C–F), indicating that in CrT cells ROS is not important in maintaining basal inflammation levels. It was also observed that silencing of SOD2 (Fig. 6G–H) and CAT (Fig. 6I–J) increased the basal level of ROS in CrT cells. These results strongly uphold the non-essential roles of ROS in the CrT cells.

3.7. Constitutively high levels of Nrf2 regulate inflammatory responses in CrT cells

To analyze the role of constitutively high levels of Nrf2 in maintaining inflammation in CrT cells, knock-down of Nrf2 was performed. Decreased expression of inflammatory markers (Fig. 7A–B) was observed. To investigate whether the high expression of Nrf2 is related to low levels of ROS in the transformed cells, we measured ROS levels after depletion of Nrf2. The low basal levels of ROS were up-regulated in cells silenced with Nrf2 shRNAs in transformed cells, as shown in fluorescence microscopy (Fig. 7C–D) These results further

corroborate the non-essential role of ROS and essential role of constitutive Nrf2 in the transformed cells.

3.8. Constitutively high levels of Nrf2 in CrT binds to the promoter regions of TNF- α and COX-2

To assess whether constitutively high expression of Nrf2 in CrT cells leads to increased binding of Nrf2 to the promoter region of COX-2 and TNF- α ChIP assays were performed. To determine the binding of Nrf2 to the ARE site of TNF- α promoter, in vitro prediction was carried out. Nucleotide sequence analysis of the 3.5-kb TNF- α promoter shows the presence of six putative AREs (Fig. 7E). ChIP assay has demonstrated the Nrf2 binding to the fourth ARE region (1374 to 1366), while other sites did not bind significantly to Nrf2. Similarly, our results also show the Nrf2 binding to the fourth ARE was increased in response to Cr(VI) treatment in the normal BEAS-2B cells, whereas the binding was dramatically enhanced in the CrT cell regardless the presence/absence of Cr(VI) (Fig. 7F). When chromatin was immunoprecipitated with control IgG, the AREs of TNF- α gene were not amplified in the same experimental conditions. These results demonstrate the direct binding of higher basal levels of Nrf2 to the promoter regions of TNF- α . Similarly, when explored the possibility of Nrf2 binding to the COX-2 promoter as mentioned (Gjyshi et al., 2014), high intensity of PCR amplification is generated due to the presence of high amount of Nrf2 in the promoter region of COX-2 (Fig. 7F). These results show that constitutively high levels of Nrf2 binds to the promoter regions of COX-2 and TNF- α , leading to increased expression of these inflammatory proteins.

4. Discussion

Previous study from our laboratory has shown that cadmium transformed cells exhibit apoptosis resistance in contrast to normal BEAS-2B (Son et al., 2014). In these transformed cells, silencing of Nrf2 increased apoptosis (Son et al., 2014; Son et al., 2015a). In the present study we have shown similar correlation in CrT cells, albeit inflammation i.e., silencing of Nrf2 resulted in decreased inflammatory response, while ROS is inessential. In contrast, acute Cr(VI) treatment in BEAS-2B cells results in increased inflammatory responses, while silencing of Nrf2 resulted in no change in the inflammation levels. These results indicate that ROS is essential in the acute Cr(VI)-induced inflammatory responses. In summary, our results indicate mechanistic differences between ROS and Nrf2 in regulating inflammatory responses before and after Cr(VI)-induced transformation.

Inflammation is implicated in Cr(VI)-induced human cancer (Beaver et al., 2009; Stout et al., 2009; Zuo et al., 2012; Thompson et al., 2014). Repetitive exposure to Cr(VI) results in persistent inflammation, which promotes tumor carcinomas (Fishbein, 1981; Malsch et al., 1994). Animal studies have shown that chromate exposure through inhalation induces lung tumors (Beaver et al., 2009). The established link between inflammation and cancer is observed in colorectal cancer, developed in patients with inflammatory bowel disease (Mantovani et al., 2008; Colotta et al., 2009). In these patients, risk of developing colorectal cancer increases from five to seven folds. Strategies for the colorectal cancer treatment target the reduction of the endogenous levels of TNF- α . In these patients, TNF- α positively

regulates NF- κ B by activating IKK. This strategy stimulates degradation of NF- κ B and enables NF- κ B nuclear translocation (Mantovani et al., 2008; Colotta et al., 2009; Dennis et al., 2009; Mantovani et al., 2010; Kamp et al., 2011). It has been reported that Cr(VI) induces inflammation by activating crosstalk between NF- κ B and AP-1 (Zuo et al., 2012). Cr(VI)-induced oxidative stress leads to increased DNA binding activities between NF- κ B and AP-1, leading to further increase in COX-2 expression (Zuo et al., 2012). Our present study demonstrates that acute Cr(VI) treatment results in an increase in expression of inflammatory markers (viz., COX-2, TNF- α and NF- κ B), which is in congruence with previously published reports on Cr(VI)-induced inflammation (Zuo et al., 2012). Cr(VI)-induced inflammation is mediated through ROS as expression of SOD2 or CAT plasmid decreased inflammation in presence of Cr(VI). These results demonstrate that ROS induced by Cr(VI) acts as upstream in the inflammatory process.

Oxidative stress and inflammation are inseparably involved in Cr(VI)-induced carcinogenesis (Pratheeshkumar et al., 2014). One of the pathways implicated in controlling inflammation is Nrf2, which is a master regulator of redox homeostasis (Kensler et al., 2007; Hayes and McMahon, 2009). Since its discovery inducible Nrf2 has been viewed as a 'good' transcription factor that protects from many diseases (Tullet et al., 2008). Studies have shown that Nrf2 activation is anti-carcinogenic in the early stage of carcinogenesis. In contrast, constitutive expression of Nrf2 is carcinogenic due to its protection of cancer cells against oxidative stress and chemotherapeutic agents (Tullet et al., 2008; Inami et al., 2011; Sporn and Liby, 2012; Son et al., 2014; Son et al., 2015b). Constitutive expression of Nrf2 is prominent in several types of human cancer cell lines and tumors (Tong et al., 2006; Inami et al., 2011). The constitutive activation of Nrf2 in CrT cells which was observed in our studies is similar to the constitutive activation of Nrf2 found in certain human cancers (Wang et al., 2008; Jiang et al., 2010). Studies have also suggested that whether Nrf2 is cancer preventive or oncogenic depends on the stages of carcinogenesis (Tullet et al., 2008; Inami et al., 2011; Sporn and Liby, 2012). As such, the biological time context is important: Nrf2 activity is desirable in the early stage of carcinogenesis, when the host is seeking to control pre-malignant carcinogenesis, but is undesirable in the later stage, when fully malignant cancer cells become resistant to apoptotic cell death (Tullet et al., 2008; Inami et al., 2011; Sporn and Liby, 2012). Our results clearly support the dual roles of Nrf2. In the BEAS-2B cells, Nrf2 does not play any role in Cr(VI)-induced inflammation. These results are consistent with previous findings that inducible Nrf2 is anti-oncogenic while constitutive Nrf2 is oncogenic (Lau et al., 2013). However, in CrT cells the inducible nature of Nrf2 is lost and constitutively activated Nrf2 supports inflammatory microenvironment. The role of Nrf2 in CrT cells is similar to stable over-expressed Nrf2 in lung carcinoma, breast adenocarcinoma, where enhanced resistance of cells towards cisplatin, doxorubicin and etoposide was reported (Tullet et al., 2008). Our studies also added more insight on the molecular events associated with increased Nrf2 in CrT cells. The constitutively high level of Nrf2 in CrT binds to the AREs of TNF- α and COX-2, resulting in increased expression of respective proteins.

Overall, our results indicate that before transformation, ROS plays a critical role while Nrf2 has no role in Cr(VI)-induced inflammation, whereas after transformation (CrT cells),

constitutively high level of Nrf2 is critical in maintaining the inflammation while ROS has no role.

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Fig. 1.

Acute Cr(VI) stress induces inflammatory responses. (A) BEAS-2B cells were exposed to increasing concentrations of Cr(VI) (1,2.5 and 5 μ M). After 24 h of exposure, total cells lysates were prepared and analyzed using Western blot to identify proteins associated with inflammatory responses (COX-2 and TNF- α) and antioxidant responsive gene markers (Nrf2). Representative Immunofluorescence images shows increased basal expression of (B) COX-2 and (C) TNF- α in Cr(VI)-treated BEAS-2B cells. (D) Total cell lysate analysis for NF κ b signaling after Cr(VI) treatment (E) cytoplasmic (NF- κ B-p65) and (F) Nuclear (NF- κ B-p65) analysis of nf- κ b signaling after Cr(VI) treatment Lamin and β -actin were used as loading control for nuclear and cytoplasmic proteins. (G) Images of subcellular localization of NF-KB-p65 under Cr(VI)-induced stress is shown. BEAS-2B cells were treated with Cr(VI) (5 μ M), and after 24 h were fixed and stained with NF- κ B-p65.

Fig. 2.

Cr(VI) stress induces ROS generation in BEAS-2B cells. (A) BEAS-2B cells were exposed to Cr(VI) at indicated concentrations for 24 h and then stained with DCFDA or DHE (10 μ M), respectively for 30 min. Cells were imaged by fluorescence microscopy. (B–C) BEAS-2B cells or BEAS-2B cells overexpressing catalase were treated with indicated concentrations of Cr(VI) for 24 h and then exposed to DCFDA (10 μ M) and subjected to flow cytometer analysis and tabulated. Data presented in the bar graphs are the mean $\pm$ SD of three independent experiments. *indicates a statistically significant difference from control cells with $p < 0.05$. (D) Electron spin resonance (ESR) spectrum recorded 15 min after addition of 5 μ M of Cr(VI) to BEAS-2B cells to measure basal level of ROS. The generation of 1:2:2:2:1 quartet ESR signal is demonstrated.

Fig. 3. ROS is responsible for acute Cr(VI)-induced inflammatory responses. Western Blot Analysis of BEAS-2B cells transiently transfected with (A) SOD2 or (B) CAT over-expressing plasmid. (C) BEAS-2B cells or BEAS-2B cells overexpressing SOD2 were treated with 5 μ M of Cr(VI). After 24 h of exposure, total cells lysates were prepared and analyzed by Western blot to identify proteins associated with inflammatory responses (COX-2 and TNF- α) and Nrf2. (D) BEAS-2B cells or BEAS-2B cells overexpressing CAT were treated with 5 μ M of Cr(VI) for 24 h and analyzed by Western blot to identify proteins associated with inflammation (COX-2 and TNF- α) and Nrf2. BEAS-2B cells or BEAS-2B cells overexpressing (E) SOD2 or (F) CAT were treated with 5 μ M of Cr(VI) for 24 and analyzed for the nuclear translocation of NF- κ B/p65.

Fig. 4. Knock-down of Nrf2 has no effect on acute Cr(VI)-induced inflammatory responses. (A) BEAS-2B cells or Nrf2 silenced BEAS-2B cells were treated with 5 μM of Cr(VI). After 24 h of exposure, total cells lysates were prepared and analyzed by Western blot to identify proteins associated with inflammation (COX-2 and TNF-α) and Nrf2. BEAS-2B cells or (B) Nrf2 silenced BEAS-2B were treated with 5 μM of Cr(VI) for 24 and analyzed for the nuclear translocation of NF-κB/p65.

Fig. 5. High basal level expression of inflammatory proteins and low levels of ROS in Chromium transformed (CrT) cells. (A) Western blot analysis of basal levels of inflammatory (COX-2 and TNF- α) and antioxidant responsive gene markers (Nrf2) in Chromium transformed (CrT) cells and in passage matched BEAS-2B cells. (B) Nuclear accumulation of NF- κ B-p65 in BEAS-2B and CrT cells. (C-D) Quantitation of basal levels of ROS in BEAS-2B and CrT cells.

Fig. 6.

ROS independent inflammation in CrT cells. Western blot analysis of CrT cells transiently transfected with (A) shSOD2 and (B) shCatalase plasmid. CrT cells silenced with (C) shSOD2 and (D) shCatalase plasmid were treated with or without Cr(VI) for 24 h, total cell lysates were prepared and analyzed using Western blot for inflammatory markers (COX-2 and TNF- α) and Nrf2. CrT cells or (E) SOD2 silenced CrT or (F) Catalase silenced CrT were treated with 5 μ M of Cr(VI) for 24 and analyzed for the nuclear translocation of NF- κ B/p65. CrT cells were transfected with (G) shSOD2 or (I) shCatalase and analyzed for ROS level. Cells were labeled with (G-H) DHE (10 μ M) or (I-J) DCFDA (10 μ M). Images were obtained by fluorescence microscopy and fluorescent intensity was determined by flow cytometry. Data presented in the bar graphs are the mean $\pm$ SD of three independent experiments. *indicates a statistically significant difference from control cells with $p < 0.05$.

Fig. 7.

Constitutively high levels of Nrf2 regulate inflammation. (A) CrT cells were transfected with shNrf2 and total cells lysates were prepared and analyzed for inflammatory markers (COX-2 and TNF- α). (B) Nuclear accumulation of NF- κ B-p65 in CrT cells after knock-down with shNrf2. (C) CrT cells were transfected with shNrf2 and basal ROS level were measured. Cells were labeled with DCFDA (10 μ M). Images were obtained by (C) fluorescence microscopy and fluorescent intensity was determined by (D) flow cytometry. Data presented in the bar graphs are the mean $\pm$ SD of three independent experiments.

*indicates a statistically significant difference from control cells with $p < 0.05$. (E). Consensus or putative ARE regions of TNF- α promoter in the 3.5 kb region. (F) ChIP analysis for increased basal Nrf2 associating with TNF- α and COX-2 in CrT cells were carried out by precipitating genomic DNA with Nrf2.