CHAPTER –II

EGFR upregulates inflammatory and proliferative responses in human lung adenocarcinoma cell line (A549), induced by lower dose of cadmium chloride
Introduction

Observation pertaining to low dose and chronic exposure of cadmium compound mentioned in the previous chapter induced lung cell proliferation along with up regulation of proinflammatory cytokines in mice. Use of anti-inflammatory drug reduced the expression of cytokines whereas cell proliferative mediators remained unchanged. We thought that there might be some mediators who monitored the cadmium induced signalling alteration in lung cells. This chapter is concerned with investigation of those particular molecules which are the cardinal regulator of cadmium induced cell proliferation.

Lung cancer is one of the leading causes of death in the world, whereas non-small cell lung carcinoma (NSCLC) accounts for approximately 75-85% of all lung cancers (Jemal et al., 2007), and most obvious correlation between cadmium and human cancer is found in the lungs (Magos, 1991, Waalkes et al., 1992; Jarup et al., 1998). Presently, the intracellular signalling pathways, involved in regulation of cellular responses induced by cadmium compounds, have received considerable attention. However, little is still known about how these events are initiated. In our previous study (Kundu et al., 2009), we have given a new insight into the relation between chronic inflammation and cell proliferation in vivo. In our present study, we showed that growth factor receptor (EGFR) is a critical mediator of cadmium acquired tolerance of A549 cells by augmenting the growth and inflammatory responses.

The EGFR is a central regulator of epithelial cell proliferation, as well as various other cellular processes in epithelial cells. Analysis of EGFR expression in human lung disease has provided evidence for its role in lung cancer. EGFR is over
expressed in 40-80% of NSCLC (Arteaga, 2003) and at least a subgroup of patients with a specific mutation in the EGFR gene have a marked clinical response to EGFR tyrosine kinase inhibitors gefitinib (Lynch et al., 2004) and erlotinib (Tsao et al., 2005). Accumulating evidences suggest that inflammation is a salutary response to insult or injury and an important part of innate immunity; however, chronic inflammation has been linked with the development of cancer (Hagemann et al., 2007). Lung injury and inflammatory responses with injurious mechanical ventilation in mice treated with pharmacologic inhibition of EGFR have already been reported (Bierman et al., 2008). On the other hand, subtoxic doses of Cd++ modulate EGF-induced DNA synthesis in a dose-dependent fashion (Enger et al., 1987). Moreover, supporting data indicated mitogenic effect of interleukin-8 on NSCLC cells, mediated via transactivation of the EGFR (Luppi et al., 2007), and EGF plays an important role in the inflammation stage of wound healing (Casacó et al., 2004). In fact, a large body of data has demonstrated that inflammatory reactions appear to be an important step in the pathogenesis of heavy metal-induced diseases (Ørrevik et al., 2006). More specifically, the expression of inducible genes leading to the synthesis of cytokines, chemokines, chemokine receptors, adhesion molecules, and autacoids plays a main role in the regulation of tumorigenesis.

Experimental evidence has implicated that cadmium produces such inflammatory responses in activated Kupffer cells, neutrophils and increased secretion of inflammatory mediators, which are involved in cadmium-induced hepatotoxicity (Horiguchi et al., 2000, Yamano et al., 2000). Previously, we proved that sublethal dose of cadmium chloride (5 mg/kg body weight), while given intraperitoneally, upregulated inflammatory cytokines such as interleukin-6 (IL-6), Cox-2 and a number of cell cycle regulatory molecules like STAT3, Akt, CyclinD1 in mice lung. It is already
reported that major downstream pathways that transduce signals from the EGFR are the RAS-p44/42 mitogen-activated protein kinase (also known as ERK1/2), PI3K-Akt and STAT3/5, furthermore, amplification and over expression of EGFRs are frequently found in a variety of epithelial cancers, such as breast, lung, colon, ovarian, and brain tumors, and play a central role in the etiology and progression of these tumors (Alroy & Yarden, 1997, Jonsen et al., 2003) The fact that was unrevealed was the key regulatory molecule responsible for both initiation of inflammation and cell proliferation in cadmium-challenged lung cell. The underlying mechanism first proved that the onset of the inflammatory and proliferative responses after cadmium treatment was due to the activation of EGFR. We showed that gefitinib, a known inhibitor of EGFR, effectively down regulated the expression of proinflammatory cytokines and cell cycle regulatory molecules.

We used A549 transformed cell line instead of a normal one because of the presence of initially higher level of proinflammatory cytokines and proliferative mediators, so that we could easily compare the fold incensement of such regulators shortly after cadmium treatment However, increased resistance to cadmium appeared in A549 cells exposed for 1 month, and over expression of stress responsive gene has already been documented (Croute et al., 2000) Moreover, it is evident that development of cadmium resistance in A549 cells unlikely results from enhanced antioxidant enzyme activities (Hatcher et al., 1995) However, the mechanism of cadmium tolerance in this transformed cell line is still not clear Here, we further investigated the potential effect of inflammatory signalling on the proliferation which might provide a novel insight into the understanding of the mechanism of cadmium resistance in various cell types
Objectives of the chapter

- To find out the key regulatory molecule who ultimately monitors cadmium induced lung cell proliferation
- To observe the effect of cadmium in lung cancer cell line

Results

2.1 Determination of LC50 and dose for experiments

Our previous study demonstrated sub lethal concentration of (5 mg/kg body weight) of CdCl₂ induced inflammation and proliferation in mice lung. We thought that it would be a high and lethal for in vitro treatment. After detailed literature review, we considered micro molar concentration of CdCl₂ for our present study (Lau et al., 2006). A549 cells were treated with varying concentration of (single dose) CdCl₂ (0, 1, 2.5, 5, 10, 20, 40, 80, 100 μM) for a short duration (72 hr). Both cell count (Fig. 2.1A) and MTT (Fig. 2.1B) assay showed that 10μM dose induced 50% of cell death (p < 0.05). Though both 2.5 and 5 μM were sub lethal concentration, we started our work with only 2.5 μM CdCl₂. Therefore, 2.5 μM concentration was chosen for further experiments to elaborate the intricate mechanisms.

2.2 In vitro wound-healing assay

After determining the dose for our experiment, we wanted to see whether 2.5 μM of CdCl₂ was able to induce proliferation or promote cell death, to evaluate our hypothesis, we performed in vitro wound-healing assay. Influence of CdCl₂ on motility of A549 cell was quantified by measuring the closure of a wound in confluent monolayer. Immediately after wounding, we took the photograph of rapid closure of
Figure 2.1. Determination of LC50 for cadmium chloride treatment. A549 cells were cultured with varying concentration of CdCl₂ (0, 1, 2.5, 5, 10, 20, 40, 80, 100 μM). After 72hr treatment, cells were collected and A trypan blue exclusion test and B MTT assay were performed. Results were representative of three independent experiments, and each value represents the percentage of live populations in each dose chosen with +/- SEM and p < 0.05, compared to normal.

Figure 2.2. A549 alveolar epithelial cells motility. Cells were suspended at 4×10⁶ cells/ml in culture medium (without serum) and was incubated at 37°C in a 5% CO₂-95% air atmosphere for 0, 12, 24, 48, and 72 hr to allow cell proliferation. It is clear from the picture that wound healing process is rapid in CdCl₂ treated cells than control one. Photographs were taken under Zeiss inverted phase microscope.
the wound of both control (H₂O treated) and CdCl₂ treated (2.5 μM) cells at different time interval (0, 12, 24, 48, and 72 h). Healing process was rapid in CdCl₂-treated cell compared with the control (Fig. 2.2). As the cells of both treated and control were allowed to grow in serum starved condition, it is clear from this experiment that the wound closure difference in A549 cell line was due to increased cell proliferation (Lee et al., 2000) by CdCl₂.

2.3 Expression of Ki-67 and PCNA

Next, to confirm whether the rapid wound closure in CdCl₂-treated cell was due to the cell migration or due CdCl₂-induced inflammation, we observed the expression of two important cell proliferative markers Ki-67 and PCNA in both control and CdCl₂-treated A549 cells. It is clear from Fig. 2.3A and Fig. 2.3B that both the expression of Ki-67 and PCNA were increased in CdCl₂ treated cells than that of the control, indicating that the rapid healing of wound in treated cells was due to increased cell proliferation rather than cell migration.

2.4 Evaluation of cell cycle pattern

Our previous data proved that low dose of CdCl₂ might induce cell proliferation. To support our wound-healing hypothesis, we treated the A549 cells with 2.5 μM CdCl₂ for 72 hr and compared the change of different phase of cell cycle with normal one. It is clear from the cell cycle phase distribution (Fig. 2.4A) and (Fig. 2.4B) that the number of cells in S phase in treated set were higher than that of the control set after 72 hr of treatment. Accumulating the results of wound-healing assay and the cell cycle phase distribution, we could suggest that CdCl₂ in low concentration augmented the cell proliferation process in transformed cell line.
Figure 2.3. Expression of cell proliferative markers and immunostaining of Ki-67. A549 cells were grown in tissue culture slides and treated with CdCl₂ (2.5 μM) for 72 hr. The cells were stained for Ki-67 as detailed in Materials and methods section. A Brown colour indicates the presence of Ki-67 protein in the nuclei of cells. Counterstaining was performed with eosin 40X. Negative control shows similar expression as control. The data shown here are from one representative experiment repeated two times with similar results. B Effect of low dose of CdCl₂ (2.5 μM) on protein expression of proliferating cell nuclear antigen (PCNA) in human lung carcinoma A549 cells. The immunoblots shown here are representative of three independent experiments with similar results. The values above the figures represent relative density of the bands normalized to β-actin (p < 0.05).
### Figure 2.4. Effect of cadmium on cell cycle distribution determined by flow cytometry analysis. B Percentage of cells were increased in S phase in treated set, while compared with the control one A which is the indication of cell proliferation. Bar graph shows the fold increase of cell cycle phases. Data represent three independent experiments (p < 0.05).
2.5 Higher dose of cadmium chloride induced cell death

Large number of evidences suggests that biphasic nature of cadmium is characterized by low-dose stimulation and a high-dose inhibition (Calabrese et al., 2008, Mattson, 2008). To evaluate this hypothesis in our experimental system, we performed scanning electron microscopy of A549 cells treated with low (2.5 μM) and higher dose (40 μM) of CdCl₂, respectively. In higher dose (Fig. 2.5A), CdCl₂ changed the morphology of the A549 cells; became round shaped, the outer membrane of the cell was disrupted, which is an induction of cell burst, and it is not the feature of the low dose and control one. The SEM data were followed by DNA fragmentation assay where A549 cells were treated with varying concentration of CdCl₂ (2.5, 10, and 40 μM) to further elaborate the biphasic character of heavy metal cadmium. It is clear (Fig. 2.5B) that DNA fragmentation induced by cadmium was concentration dependent; DNA fragmentation increased with increasing concentrations of CdCl₂ up to 10-40 μM after 72hr exposure. However, no fragmentation was seen in control and lower dose of CdCl₂ (2.5 μM).

2.6 Initial inflammatory responses induced cell proliferation via EGFR up regulation

As we mentioned earlier that EGFR activation could lead to the development of severe inflammatory responses (Bierman et al., 2008) and on the other hand, cadmium compound induced DNA synthesis via the activation of EGFR in a dose-dependent manner (Enger et al., 1987), we then focused to find out whether there was any correlation between cadmium-induced proliferations and inflammation with EGFR activation. For searching out the intrinsic mechanism, we used gefitinib, known inhibitor of EGFR, and observed the expression status of different cytokines.
Figure 2.5. Scanning electron microscopy and DNA ladder assay of cadmium-treated A549 A High CdCl₂ concentration damages the cellular surface of A549 cells (300X) No such cellular damage was observed in control and low dose treated set (100X) B DNA ladder assay proved that fragmentation of DNA of A549 cell was increased due to increasing concentration of CdCl₂ The latter observation is representative of three independent experiments
Figure 2.6 Effect of cadmium chloride on the expression of cytokines and cell cycle regulatory molecules

A CdCl₂ selectively upregulated the expression of tumor necrosis factor-α (TNF-α) at both 24 and 48 hr, whereas B IL-1β expression was only significant at 48 hr. No further increases in the expression of these cytokines were observed at late stage (data not shown). In contrast, the expression P-Akt and P-STAT3 were very low at early stage but were increased with the time course. EGFR expression was moderate throughout the experimental period. C Bar graph shows the relative expression of respective proteins. D Gefitinib effectively inhibited phosphorylation of P-Akt, P-STAT3, and reduced the levels of EGFR and the cytokines. A and B indicated the regulation of EGFR on such mediators. Data represent three independent experiments (p < 0.05).
by ELISA and different cell cycle regulatory molecules, known to be regulated by EGFR. We found that in early stage of cadmium exposure (24 and 48 hr) TNF-α and IL-1β (Fig. 2.6A) and (Fig. 2.6B) were up regulated, but the expression status of other downstream molecules of EGFR like P-STAT3 and P-Akt remained at basal level (Fig. 2.6C). We also investigated the expression status of the same regulatory molecules in late hour (72 hr) of CdCl₂ exposure and interestingly, we found increased expression of EGFR, P-STAT3 and P-Akt (Fig. 2.6C), but the expression of the cytokines were not changed significantly (data not shown). On the other hand, gefitinib effectively decreased the expression level of cytokines in early stage and cell cycle regulatory molecules in late stage (Fig. 2.6D) All these accumulating evidences proved the involvement of EGFR in early inflammatory responses in CdCl₂ challenged lung carcinoma cell line, which might be responsible for the late stage cell proliferation.

2.7 IL-6 was also up regulated in cadmium-treated cells

Our immunofluorescence study showed that increased expression of IL-6 at early stage of CdCl₂ challenge (Fig. 2.7), but the expression remained static in subsequent 48 and 72 hr (data not shown) The interesting observation was that gefitinib altered the expression profile of IL-6, indicating the fact that in CdCl₂ challenged A549 cell, up regulation of IL-6 along with the other cytokines remain under the control of EGFR

2.8 Gefitinib inhibited the expression of CyclinD1 and Cox-2

In normal and cancer cells, the G1/S-phase transition of the cell cycle is dependent on signal-induced expression of both early genes and D-type Cyclins. To
Figure 2.7. EGFR regulated the expression of IL-6. A549 cells were grown on cover slip; after 24 hr treatment, cells were prepared for immunofluorescence study of IL-6. DAPI stained the nucleus, whereas green fluorescence by FITC conjugated anti IL-6 antibody indicated over expression of IL-6 in cytoplasm. Expression was also markedly inhibited by gefitinib.
Figure 2.8. Expression of Cox-2 and CyclinD1. The cell lysates from A549 cell were prepared, and the expression level of Cox-2 and CyclinD1 were analyzed. The expression of both the protein was inhibited when gefitinib was present. Data represent three independent experiments (p < 0.05).
date, it is already established that EGFR cross signalling leads to shared transcriptional responses regulating the expression of CyclinD1 (Kalish et al., 2004). In our cell cycle study, we found increased cell number in S phase. On the other hand, Cox-2 expression is sometime mediated through the EGFR activation (Chien et al., 2006), and Cox-2 is an inducible inflammatory enzyme that plays an important role in the progression of human lung adenocarcinoma (Wolff et al., 1998). It has also been reported that IL-6 and its receptor interactions activate STAT3 which in turn induce the expression of several anti apoptotic proteins and thereby promotes cell proliferation. It is documented that cell expresses elevated levels of CyclinD1 when stably transfected with a dominant-active STAT3 construct (Simbaldi et al., 2000). So, we wanted to see whether EGFR inhibition would have any effect on these downstream mediators, and we found gefitinib effectively blocked the expression of Cox-2 and CyclinD1 in late hour of CdCl₂ (Fig. 2.8) and established the fact that CdCl₂ induced inflammatory and cell proliferative responses were linked through EGFR mediated pathway.

Discussion

Although various reports have demonstrated that cadmium associated cytotoxicity is intimately related to apoptosis, investigations into the signalling mechanisms of this process have yielded different results. Evidences are there indicate that low dose of cadmium can induce neoplastic transformation of human prostate epithelial cells (Bakshi et al., 2008). Cadmium-induced tumours of the testes and pituitary at the injection site in the Nobel rat in a dose-dependent fashion (Wâalkes et al., 1999). In addition to these overt carcinogenic effects, cadmium treatment also induced presumptive and pre-neoplastic proliferative lesions in the dorsal lobe of the
consistent with this, we previously determined that cadmium cytotoxicity
induced inflammation and proliferation in lung, in vivo system. Several studies
have been demonstrated in connection with the mechanism of cadmium-induced cell
death, but no such evidences have been found till date indicating the molecular
interplay behind the cadmium and cell proliferation.

Our previous study demonstrated clearly that cadmium induced significant cell
proliferation and inflammation at low concentrations and inhibited cell growth at high
concentrations, but at the same time, no molecular mechanisms underlying such
biphasic effect of cadmium were reported. Although cell proliferation at low
concentrations of cadmium can be blocked to some extent by the inhibitors of
ERK1/2 and JNK, the decreases of cell proliferation at high concentrations of
cadmium were restored by p38 inhibitor and have been documented (Jiang et al.,
2009). In this study, we aimed to determine the cross talk between the onset of
inflammation and its relation to ultimate cell proliferation by cadmium exposure in
A549 cell, which has not been evident so far.

We found that EGFR expression is critical for the up regulation of
proinflammatory cytokines like TNF-α, IL-1β and IL-6 in early stage of cadmium
challenged, which results in the late stage cell proliferation. Considering altogether,
low concentration of cadmium may activate the EGFR which in turn induces two of
its downstream mediators STAT3 and Akt. We know that EGFR plays an important
role in the regulation of cell proliferation, differentiation, development, and
oncogenesis (Mendelsohn and Baselga, 2006). EGFR is expressed at high levels in a
number of tumour types and in most lung cancer. In this study, due to cadmium
treatment, A549 cells constitutively expressed EGFR which may contribute to the
resistance of these cells to apoptosis in lower concentration. Over expression of EGFR activates PI3K, and Akt has been reported to be associated with the proliferation and survival of cells (Rexer et al., 2009) Regarding the effects of EGF on downstream pathways in cells transfected with wild type and mutant EGFR, Sordella et al. reported that Akt and STAT5 were phosphorylated in serum-starved culture conditions in L858R cells (Sordella et al., 2004)

We showed that the major distinct feature of EGFR expression promoted inflammatory responses along with cell proliferation, which was further confirmed by the use of gefitinib. Here, we have shown that in early stage of cadmium exposure, inflammatory cytokine expression were up regulated and the effect was limited for a short period of time (48 hr), but as long as the cadmium remained in the culture medium, it induced cell proliferative regulators such as P-Akt, P-STAT3, CyclinD1 and Cox-2 along with EGFR. These results suggest that cadmium, by activating EGFR, produces inflammatory microenvironment at early stage and immediately switched to the induction of cell proliferation if it was allowed to stay in cell for longer period of time.