CHAPTER-I

Cadmium induces lung inflammation independent of lung cell proliferation: a molecular approach
Introduction

Cadmium has been shown to have various detrimental effects on health (Jarup et al., 1998). Upon absorption, Cadmium is rapidly transported by blood to different organs in the body where its estimated half-life in humans is 15-20 years (Jin et al., 1998) Additionally chronic exposure to cadmium has been associated with a number of physiological consequences such as renal failure and immunosuppression as well as various types of cancers in mammals. Several toxicities such as hepatotoxicity, neurotoxicity and cardiotoxicity are also documented under high cadmium exposure (Rikans et al., 2000; Thevenod, 2003) In recent years progress has been made in dissecting apart the molecular mechanisms underlying the effects of exposure to this toxic metal

The amount of cadmium absorbed in the body following its exposure varies depending on the route of entry Though the primary routes of cadmium exposure in humans are via inhalation from such sources as cigarette smoking (Nandi, 1969), food is also reported as source for human exposure to cadmium Cadmium is selectively taken up by certain edible plants and certain food items, such as crab contains cadmium as high as 30-50 ppm (Schwartz et al., 2000) In general, exposure of cells to low, micromolar concentrations of cadmium results significant toxicity (Othumpangat et al., 2005, Badisa et al., 2008) Strong evidence, based on experimental studies exists to support the carcinogenic potential of cadmium Following various routes of exposure to cadmium, experimental animals produce tumours of multiple organs (Waalkes et al., 1989, Waalkes et al., 1999) Only about 5% of a given dosage of cadmium is absorbed from the gastrointestinal tract while lung absorption is as much as 90% of a dose inhaled into the lungs Despite being one
of the major routes for cadmium absorption, the toxic mechanism of cadmium on lung tissue is still poorly understood (Oh et al., 2004).

Cadmium induced lung injuries have been recently identified which indicates that it provokes lung damage and inflammation (Bell et al., 2000) by involving cytokine production (Kataranovski et al., 1998). Cadmium-adapted alveolar epithelial cells are protected from oxidant-induced apoptosis along (Eneman et al., 2000) with the expression of the numerous genes in acute-phase proteins or inflammatory cytokines (Harstad et al., 2002). The acquired self tolerance to cadmium is thought to have some basis in toxicokinetics but primarily concerns with modified tissue responses (Klaassen et al., 1999)

Cadmium is one of the inflammation related xenobiotics and its exposure on the tissues is often accompanied with infiltration of inflammatory cells (Kuester et al., 2000). Interleukin, such as IL-6 has a key role in the proliferation of lung cell (Bihl et al., 1998) and Cox-2 is an inducible inflammatory enzyme plays an important role in the progression of human lung adenocarcinoma (Wolff et al., 1998) Although Cox-2 expression in tumors increases angiogenesis, which is highly associated with induction of various growth factors like IL-6 (Jee et al., 2001) Various studies reported that cadmium promotes lung cell proliferation by an immune suppressive network which involves over expression of Cox-2 (Khuri et al., 2001) On the other hand 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 reported that cell expresses elevated levels of CyclinD1 when stably transfected with a dominant active STAT3 construct (Simbaldi et al., 2000) Therefore the general mode of action of all these signalling molecules are either directly related to the inflammation only or to the development of cell proliferation influenced by
chronic inflammation. Although cadmium exposure has been reported to cause neoplastic transformation of human prostatic epithelial cells, but the efficacy of this transformation is highly dependent on the dose of the metal ion (Achanzar et al., 2001, Nakamura et al., 2002) Exposure of normal human prostate epithelial cells to higher dose (10μM) cadmium transiently increased the expression of p53, c-myc, and c-jun after 2 hr as a prelude to apoptosis (Achanzar et al., 2000) where as lower dos promotes proliferation and resistance to apoptosis

We are particularly interested in the contribution of low doses (5 mg/kg body weight) of cadmium to the transformation of mice lung. We have found that, due to cadmium exposure expression of IL-6, STAT3 and inflammatory enzyme MMP-2, Cox-2 increased significantly Also we have the evidences that increased activity of the Akt signalling axis in lung cells appears to operate in conjunction with or parallel to increased STAT3 activation to induce proliferation programme We showed that cadmium promotes lung inflammation and cell proliferation both in independent manner Thus, this study was designed to determine the effects of chronic exposures of low concentration of cadmium in vivo It is not worthy that anti-inflammatory drug treatment could not totally inhibit the proliferation process, whereas inflammation was prevented

Objectives of the chapter

✓ To observe the effect of low concentration of cadmium, whether it causes lung cell proliferation or cell death?
✓ To investigate the cellular mediators through which cadmium alters the cellular functions.
✓ To evaluate whether cadmium induced inflammation is responsible for lung cell proliferation or not.
Results

1.1 Determination of LD50 and dose for experiments

To determine the LD50, mice were administered different concentration of cadmium chloride (CdCl₂) (2.5, 5, 10, 20, 40 and 80 mg/Kg body weight) till 60 days period (single dose in a week). It was found that 50% of the experimental population died at a concentration of about 10 mg/Kg body weights, (Fig. 1.1) and there was a significant decrease (p <0.05) of survival according to the increasement of dose Therefore 5 mg/Kg body weight (sub lethal dose) concentration was chosen for further experiments to elaborate the intricate mechanisms.

1.2 Prolonged exposure of low dose of Cadmium induces lung oedema and inflammation

To evaluate the effect of low concentration of CdCl₂ on mice lung cell, we pulsed the mice with 5mg/kg body weight CdCl₂ for different time interval subsequently for 15 days, 30 days, 45 days, and 60 days No lung lesion or oedema was shown to develop up to 45 day Histological analysis revealed that appearance of alveolar oedema and inflammation along with the airspace enlargement after 8-week of CdCl₂ exposure (Fig. 1.2B) At the same time, mice those who were exposed to same dose of CdCl₂ along with non steroidal anti-inflammatory drug like Ibuprofen (50mg/kg body weight), showed reduced level of inflammatory development (Fig.1.2C) while compared to the normal (Fig. 1.2A) This data suggests that CdCl₂ if applied even in a very low concentration for a long period of time can induce inflammation in experimental animals
Figure 1.1. Dose dependence survival of Cadmium treatment Each value represents the percentage of died populations in each dose chosen with +/- SEM (n = 5) and p < 0.05, compared to normal. The experimental groups represent the Cadmium treated mice [inject CdCl₂, (i.p.)] at a concentration of 2.5 mg/Kg body weight, 5 mg/Kg body weight, 10 mg/Kg body weight, 20 mg/Kg body weight and 40 mg/Kg body weight and 80 mg/Kg body weight respectively. The sub lethal concentration (5 mg/Kg body weight) was chosen as an applied dose.
Figure 1.2. Histopathology of lung sections of Swiss albino exposed to cadmium (5 mg/Kg body weight) showing progressive lung inflammation and prevention by Ibuprofen. A, Histology of lung sections of normal mice. B, Mice were treated with low dose of CdCl₂ (5mg/Kg body weight), subsequently for 15, 30, 45, 60 days, granulation and air space enlargement was shown after 45 days which is the sign of inflammatory development. C, Application of Ibuprofen reduces the chance of lung oedema formation throughout the experimental periods. Original magnification (100X).
1.3 Zymography proves MMP-2 but not MMP-9 is one of the mediators of such type of inflammation

There are different mechanisms are thought to be responsible for the development and progression of cadmium induced inflammation in lung. Increased secretion and/or activity of matrix metalloproteinasmes (MMP), especially MMP-2 and MMP-9, have been identified in inflammatory cells and tissues isolated from human suffering from COPD (Atkinson et al., 2003) In order to find out the mechanism of CdCl₂ induced lung inflammation we performed the zymograph analysis to measure the matrix metalloproteinase expression. We observed significant expression of MMP-2 but not MMP-9 (Fig.1.3) throughout the experimental period. Though NSAID like Ibuprofen is sufficient enough in suppressing the expression of matrix metalloproteinases, but it failed to operate its inhibitory action here, p < 0.05 with respect to the control.

1.4 Cyclooxygenase-2, IL-6, P-STAT3 and P-Akt expression are the key regulators of cadmium induced lung inflammation

The regulation of Cox-2 is very important in various inflammatory and proliferative responses, which is sometime induced by IL-6 (Jee et al., 2001) signalling pathway Therefore, we wanted to see that whether this CdCl₂ induced lung cell proliferation has any relationship with Cox-2 and IL-6 expression and we found that expression of both Cox-2 and IL-6 were increased to a significant level, while application of Ibuprofen was able to reduce the expression of Cox-2 and IL-6 (Fig.1.4A) and it was strongly supported by our ELISA results (Fig. 1.4C) STAT3 and Akt are the downstream modulators of the IL-6 and there regulations are important in growth signalling. So we studied the expression of P-STAT3 and P-Akt,
Figure 1.3. SDS-PAGE and Gelatin zymography represent the expression of MMP-2. Detection, by gelatin zymography, of matrix metalloproteinase-2 (MMP-2) and (MMP-9) expression at different time points after the induction of CdCl2 (5mg/Kg body weight). Lung cell extracts were prepared at days of 15, 30, 45 and 60 from normal (N), CdCl2 treated and CdCl2 plus Ibuprofen treated (three individual animals per dose) mice. The zymography was developed and stained as described in Materials and Methods. The picture shows increased expression of MMP-2 (72kD), which was not inhibited by Ibuprofen. Gel is representative of three comparable experiments indicate p < 0.05 with respect to the control.
Figure 1.4. Cadmium induced expression of Cox-2, IL-6, P-STAT3 and P-Akt. Cell lysate from control and treatment lung were subjected to western blot analysis and ELISA. A, Expression of Cox-2, IL-6, P-STAT3, P-Akt were increased throughout all the experimental period. Though Ibuprofen reduced the expression of Cox-2 and IL-6, but it could not be able to revert the expression of P-STAT3 and P-Akt. B, Bar graphs represent the quantitative densitometric value of the expressed protein. C, OD values showed significant increasement of Cox-2 and IL-6 expression by ELISA. Data is representative of three comparable experiments and indicate p < 0.05 with respect to the control.
following CdCl₂ exposure. In our study, we also observed that both P-STAT3 and P-Akt expression (but not the normal STAT3 and Akt) were upregulated (Fig. 1.4A) in treated mice, but application of Ibuprofen could not revert these expression statuses. The result indicated that CdCl₂ induced cell proliferation through P-STAT3 and P-Akt activation may have inflammation independent pathways. Data is representative of three independent experiments (p < 0.05).

1.5 Immunohistochemical expression of Cox-2

To support our previous result, we observed the expression level of Cox-2 by immunohistochemistry. After two months of exposure, CdCl₂ treated lung showed increased level of Cox-2 expression (Fig. 1.5B), and was reduced in Ibuprofen treated lung (Fig. 1.5C), while compared with the control slide (Fig. 5A). In negative control set, no such expression of Cox-2 was observed (Fig. 1.5D, 1.5E, 1.5F). We observed the same result in case of IL-6 expression (data not shown). The result proved that low dose of cadmium causes inflammation of lung cell by inducing the proinflammatory cytokine like Cox-2 and IL-6.

1.6 Low-dose Cadmium exposure increased cell number

So far we have proved that CdCl₂, when applied in a low concentration (sub-lethal dose), capable of inducing the expression of some proteins, those are very much involved in stimulating cellular inflammation and proliferation. Therefore, we took the lung cell count after the exposure of different concentrations of CdCl₂. We found that cell number increased in mice those were adapted with low dose of CdCl₂ (5 mg/Kg body weight), but at the same time reduced cell count were followed in mice treated with high dose of CdCl₂ (10 mg, 20 mg, 40 mg and 80 mg/Kg body weight).
Figure 1.5. Immunohistochemical expression of Cox-2. A, Control lung shows no expression of Cox-2. B, Cox-2 expression was profoundly increased in treated lung. C, Application of Ibuprofen reduces the expression level of Cox-2. D, E, F, Negative staining shows no such expression. Original magnification (100X).
Figure 1.6. Cadmium causes lung cell proliferation. We evaluated the effect of CdCl₂ on the lung cell count in normal and treated mice. The normal and treated mice were given pyrogen free saline water and different dose of CdCl₂ (2.5 mg, 5 mg, 10mg, 20 mg, 40 mg and 80 mg/Kg body weight) respectively for two months. Cell count gradually increased in low dose of CdCl₂ (2.5, 5 mg/Kg body weight), but beyond that (10 mg, 20 mg, 40 mg and 80 mg/Kg body weight) cell death occurred. Data is representative of three independent experiments (p < 0.05).
When compared with the normal (Fig. 1.6), this data clearly indicates that chronic CdCl₂ exposure first induced inflammation and then cell proliferation. Data is representative of three independent experiments (p < 0.05).

1.7 DNA damage analysis clearly suggests that cellular effect of cadmium is totally dose dependent.

For further confirmation of our cell count data, we performed single cell gel electrophoresis (Comet assay), and found that low exposure of CdCl₂ (5 mg/kg body weight) did not show any significant DNA damage. We performed the assay also with high dose (80 mg/kg body weight) of CdCl₂, to see whether it shows the same effect or not. We observed that 80 mg/kg body weight CdCl₂ induces tail formation, which was the indication of DNA damage. The result proves dual role of CdCl₂, when applied in low dose it causes cell proliferation, otherwise it promotes cell death (Fig. 1.7A). It is clear from the Fig. 1.7B and Fig. 1.7C, that tail formation and cellular deformity were increased along with the application of high CdCl₂ concentration. Data is representative of three independent experiments (p < 0.05).

1.8 Evaluation of cell cycle pattern after cadmium treatment

We next studied the cell cycle phase distribution of lung cells treated with CdCl₂ (5 mg/Kg body weight) for two months (Fig. 1.8). It is interestingly seen that the results supported our notion and the total DNA content of S-G2/M phase in treated (Fig. 1.8B) group increased by 31.91% compared to that of 11.40% in normal one (Fig. 1.8A). To further confirm this data, we examined the DNA laddering pattern of the treated lung and compared it with the control set. It is clear from the Fig. 1.8C that no the ladder was formed in the low CdCl₂ treated lung, which can be correlated.
Figure 1.7. Comet assay. A, DNA tail formation was increased in higher dose of CdCl$_2$, while no significant change was observed between control and low dose. B, C, Showed tail formation increased significantly in 80mg/Kg body weight dose of CdCl$_2$. Data is representative of three independent experiments (p < 0.05).
Figure 1.8. Effect of cadmium on cell cycle distribution of mice lung cell determined by flowcytometry analysis. **B.** Percentage of cell increased in S-G2/M phase in treated set. **A.** Increased cell number was observed in sub-G0/G1 phase in control set, which was the indication of cell proliferation. Data is representative of three independent experiments (p < 0.05). **C.** No DNA ladder was formed in low dose (5 mg/Kg body weight) CdCl₂ treated lung while compared with control ladder (L) and normal one.
with our cell count data and it strongly suggests that CdCl₂ (low dose) itself by promoting inflammatory responses can produce cellular proliferation in mice lung, which could not be reverted by non steroidal anti-inflammatory drug like Ibuprofen (data not shown). Data is representative of three independent experiments (p < 0.05).

1.9 Scanning Electron Microscopy

Our cell count and cell cycle results clearly indicated that instead of inducing cell death mechanism CdCl₂ in low concentration promotes cell proliferation. We observed the surface structure of lung after 60 days by scanning electron microscopy to see whether there is any formation of tumour likes growth or not. We did not observe any tumour pattern on cell surface (Fig. 1.9A), but architectural distortion of the pulmonary microvasculature was evidenced in treated mice. Alveolar space was getting narrowed along with the enlargement epithelial basement (Fig. 1.9B), which is the indication of acute inflammation. In higher magnification, cell surface became uneven and lobular (Fig. 1.9C), Narrow edicular spaces signify the increase of cell density (Fig. 1.9D). Ibuprofen showed no effect to revert the structural deformity of lung caused by CdCl₂.

1.10 Cadmium Induced CyclinD1 Regulation in lung Cell

Our previous experiment has proved that CdCl₂ (5mg/Kg body weight) challenged lung cell acquired the capacity of proliferation after a chronic exposure. Our cell cycle pattern has revealed that cell number is increased significantly in S and G2/M phase along with the reduction in G0/G1 phase, which is a clear indication of cell proliferation. Therefore, we had to determine the expression of any regulator,
Figure 1.9. Scanning Electron Microscopy of cadmium treated lung  

A. No lobular appearances were observed under lower magnification B. Alveolar space was getting narrowed in treated lung, compared to the normal one C. Lobular appearances were prominent at higher magnification D. Narrow edicular spaces signify the increase in cell density Ibuprofen showed no effect to revert the structural deformity
Figure 1.10. Effect of cadmium on CyclinD1 expression. A, Expression of CyclinD1 was increased throughout all the experimental period. B, Bar graphs represent the quantitative densitometric value of the expressed protein. Data is representative of three comparable experiments indicate p < 0.05 with respect to the control. C, Immunoprecipitation showed the formation CyclinD1-Cdk4 complex, which is an indication of cell cycle progression.
which is very much related with this phage (S and G2/M) of cell cycle. We know that CyclinD1 can promote both G0/G1/S and S/G2/M progression (Cai et al., 2006). Our western blot data shows that expression of CyclinD1 is increased in treated mice (Fig. 1.10A), for further confirmation we immunoprecipitated the cell lysate first with Cdk4 antibody, and the performed SDS-PAGE with anti CyclinD1 antibody. Fig. 1.10C indicated the complex formation of Cdk4-CyclinD1 which was an evidence of cell cycle progression. Data is representative of three independent experiments (p < 0.05).

Discussion

In this study we tried to reveal the mechanistic details of cadmium induced inflammation and proliferation in lung. An association between the development of cancer and inflammation has long been appreciated (Folkman, 2002). The chronic inflammatory states associated with infection and irritation may lead to environments that foster genomic lesions and tumour initiation (Rakoff-Nahoum, 2006). The results of the present study showed that chronic exposure of cadmium compound induces lung cell proliferation which may be independent of lung inflammation. We hypothesized that cadmium exposure induces the inflammatory cytokines along with the cell proliferating factors in the lungs of mice.

We found that cadmium causes cell death at high concentration but at low level it is capable of inducing proliferation. Evidences are there indicate that low dose of cadmium can induce neoplastic transformation of human prostate epithelial cells (Bakshi et al., 2008). Lung epithelial cells of cadmium treated (5mg/Kg body weight) mice exhibits elevated level of cellular proliferation along with the accumulation of inflammatory molecules and cytokines. Therefore, in search of the mechanism behind
it we found that cadmium induced the cellular signals to shift towards the proliferation as a whole, but prior to the development of cell proliferation cadmium initiated severe lung inflammation. There is a supportive evidence that, lung cell is able to become gradually resistant in response to cadmium (Lau et al., 2006) We observed that IL-6 and inducible inflammatory enzyme Cox-2 elevated significantly in cadmium exposed mice. We also observed the expression level of TNFα, IL-1β, Hsp70, in cadmium treated lung cell (data not shown), but we did not find any significant change in their expression. That is why we mainly focused to see the role of cadmium on influencing the cellular expression of Cox-2, IL-6 and their downstream mediators, because of the fact that along with inflammation, these are the two known parameters of tumor development. Currently, Cox-2 inhibitors are being assessed in clinical trials for chemoprevention and as an adjuvant for conventional therapy in lung cancer (Harris et al., 2007). Additionally, anti-IL-6 therapy has shown promising results in metastatic condition (Tassone et al., 2005) In the current study, we showed that elevated IL-6 and Cox-2 expression could be reduced by non steroidal anti-inflammatory drug like Ibuprofen

Morphological differences between lungs of control and treated mice clearly suggest the development of oedema In inflammatory setting the inducible Cox-2 are detected in various reports (Peppelenbosch et al., 1993) We showed it also through ELISA and immunohistochemistry In our consideration lung cells switch on such signalling which helps to proliferate against the stressed environment. It is already suggested by various scientists that IL-6-activated Janus kinase which leads to the activation of signal transducer and activator of transcription (STAT) and Akt signalling cascades (Cabrespine et al., 2007) There are supportive evidences that cadmium increases IL-6 production (Brama et al., 2007) and Akt activation in cancer
cell (Chen et al., 2006) On the other hand IL-6 induced ICAM-1 expression is mediated via JAK/STAT signalling pathway in which STAT3 phosphorylation followed by its binding to IRE which are in the promoter of cell cycle related genes including CyclinD1. Constitutive activation of STAT3 signaling contributes to oncogenesis by preventing apoptosis and enhancing cell proliferation. Moreover, Cox-2 dependent expression of IL-6 has been implicated in STAT3 activation and IL-6-dependent STAT3 activation has been shown to increase angiogenesis in several cancers (Dalwadi et al., 2005). We showed that though Ibuprofen reduces the expression level of Cox-2 and IL-6, it could not prevent the expression of P-STAT3 and P-Akt and CyclinD1. So, due to chronic exposure, cadmium promoted inflammation independent cell proliferation. Our flowcytometric results suggest cell cycle progression. It has been reported that STAT3 activation up regulates target genes, such as CyclinD1, that leads to cell cycle progression or prevention of apoptosis and STAT3 inhibition results in the down regulation of CyclinD1 (Masuda et al., 2002). As we know that CyclinD1 is responsible for G0/G1/S and S/G2/M transition (Cai et al., 2006), therefore, we have correlated both of the background information to test the expression of CyclinD1 and found that CyclinD1 was up regulated in lung cell after cadmium treatment, suggesting these pathways may operate in our system. Collectively, these findings suggest an important role for IL-6, Cox-2, STAT3, Akt and CyclinD1 in cadmium induced inflammation and lung cell proliferation.

We exposed mice to different concentrations of cadmium and found that low levels of cadmium (5 mg) consistently increased cell viability but higher levels of cadmium inevitably led to cell death with same exposure. A similar biphasic response has been reported previously by in vitro study (Achanzar et al., 2000). So it is the first
in vivo study which might provide a mechanistic explanation for cadmium-induced cell proliferation independent of inflammation in normal lung cells. Many of the observations described cells are adapted when cadmium exposure continued for a longer period (Waisberg et al., 2003). Similar observations of chronic pulmonary inflammation reported in cigarette smoke exposed mice (Seagrave et al., 2004). We proved that for the first time that cadmium exposure promotes pulmonary inflammation but this may not be the cause of lung cell proliferation.