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PLAGL2 translocation and SP-C promoter activity—A cellular response of lung cells to hypoxia

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Abstract

Cobalt is a transition metal which can substitute for iron in the oxygen-sensitive protein and mimic hypoxia. Cobalt was known to be associated with the development of lung disease. In this study, when lung cells were exposed to hypoxia-induced by $CoCl_2$ at a sub-lethal concentration (100 μM), their thyroid transcription factor-1 (TTF-1) expression was greatly reduced. Under this condition, SP-B promoter activity was down-regulated, but SP-C promoter remained active. Therefore, we hypothesized that other factor(s) besides TTF-1 might contribute to the modulation of SP-C promoter in hypoxic lung cells. Pleomorphic adenoma gene like-2 (PLAGL2), a previously identified TTF-1-independent activator of the SP-C promoter, was not down-regulated, nor increased, within those cells. Its cellular location was redistributed from the cytoplasm to the nucleus. Chromatin immunoprecipitation (ChIP) and quantitative RT-PCR analyses demonstrated that nuclear PLAGL2 occupied and transactivated the endogenous SP-C promoter in lung cells. Thereby, through relocating and accumulating of PLAGL2 inside the nucleus, PLAGL2 interacted with its target genes for various cellular functions. These results further suggest that PLAGL2 is an oxidative stress responding regulator in lung cells.

Keywords: PLAGL2; TTF-1; Differential regulation; Relocation; Translocation; SP-C; SP-B; Cobalt chloride; Hypoxia

Surfactant proteins B (SP-B) and C (SP-C) are two small hydrophobic proteins, essential for maintaining surface tension in pulmonary alveoli. The regulation of SP-B and SP-C genes is attributed to the binding of thyroid transcription factor-1 (TTF-1), a homeodomain transcription factor, to their promoters [1,2]. For SP-C, its cell specificity is controlled within the proximal region of the SP-C promoter, which contains two essential TTF-1 binding sites (T4 and T5) and a previously identified cis-element for pleomorphic adenoma gene like-2 (PLAGL2) binding [3]. Thus, besides TTF-1, PLAGL2 was suspected to be another modulator of the SP-C promoter. The cis-element for PLAGL2 binding on the SP-C promoter is functional [3]. In addition, transfection studies in lung and non-lung cells further indicated that PLAGL2 activation of the SP-C promoter was independent of TTF-1 and other lung cell-specific factor(s) [3].

PLAGL2 was first identified by homology to PLAG1, a zinc finger protein [4]. PLAG1, PLAGL2, and another PLAG-like protein, PLAGL1, form a PLAG gene family. Both PLAGL2 and PLAG1 transform NIH3T3 cells [5]. However, they induce apoptosis rather than proliferation in other types of cell [6], suggesting a versatile role of PLAGL2 in cell cycle regulation. In addition, PLAGL2 could be induced by hypoxia or "hypoxia-mimics"—namely cobalt chloride ($CoCl_2$) in some cells [7].

Cobalt, one of the transition metals, can substitute for the iron atom in the heme protein which binds oxygen. The mechanism by which Co^{2+} activates hypoxia inducible factor-1 (HIF-1) and mimics hypoxia in cells may be by substitution for Fe^{2+} in the regulatory dioxygenase to inactivate the enzyme [8]. In lung cells, the underlying mechanism of cobalt-induced pneumotoxic effect was suggested by the down-regulation of the negative regulators of

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HIF-1 [9]. Though cobalt has been known to associate with the development of lung interstitial fibrosis disease, there is little known about its influence on surfactant protein genes expression. In this study, we showed that $CoCl_2$-induced oxidative stress down-regulated SP-B promoter activity by repressing TTF-1 expression. On the other hand, the SP-C promoter stayed active despite the reduction of TTF-1, raising a possibility of other factor(s) contribution to the promoter activity. Given that PLAGL2, a previously identified SP-C promoter activator, was not down-regulated but relocated and accumulated in the nucleus, a role of PLAGL2 in preventing the loss of SP-C-related lung function by hypoxia was suggested.

Materials and methods

Cell culture. MLE12 cells (murine type II cells, ATCC CRL-2110) and H441 cells (human lung adenocarcinoma cells, ATCC HTB-174) were maintained as previously described [3,10]. For immunofluorescent study, cells were seeded overnight before being treated with 100 μM $CoCl_2$ for 48 h. Both treated and untreated cells were then fixed in situ by 4% paraformaldehyde in PBS, permeabilized by 0.1% saponin, and probed with the monoclonal antibody to PLAGL2 (mAb85C47-1). Rabbit anti-mouse-FITC antibody was used as the secondary antibody for the detection and was observed under a fluorescent microscope.

Chromatin immunoprecipitation assay (ChIP). ChIP assay was performed using a protocol exactly as previously described [10]. The SP-B or SP-C promoter fragments were generated by PCR using the following primers: SP-B promoter, 5′-primer GTTTGACGGTGAACAAAGTCA GGCT and 3′-primer GACCTCAGTGTTTGCCTGTGTCT for mouse, 5′-primer CCAGGAACATGGGAGTCTGG and 3′-primer TAGGAGT GGCAGCGACCTC for human; SP-C promoter, 5′-primer GGCAGAC ATGCAGAAAGACA and 3′-primer TCCTTGGCTTTGTAGCTTGTT for mouse, 5′-primer CCCGAGGGCAAGTTTGCTC and 3′-primer CA AGCCCTTGGCTTTGAAGC for human. The program for PCRs was: 95 °C, 1 min; 59 °C, 1 min; and 72 °C, 1 min.

Western blot analysis. Cellular proteins obtained from cell lysates used in the luciferase assay were fractionated on a 10% SDS–PAGE gel and then transferred to a PVDF membrane for Western blot analysis. The membrane was probed with antibodies to PLAGL2 (mAb85C47-1, 1:3000), anti-TTF-1 antibody (Zymed, South San Francisco, CA; 1:3000), or anti-actin monoclonal antibody (Chemicon, Temecula, CA; 1:10,000) followed by goat anti-mouse antibody conjugated with horseradish peroxidase (HRP) (Pierce, Rockford, IL; 1:50,000) and then developed with HRP Supersignal substrate (Pierce, Rockford, IL).

In vitro transfection and plasmids. Transfection assays were conducted as described in the previous manuscripts [10,11]. Each data point presented in the transfection studies was collected from at least three individual experiments with triplicates ($N \geqslant 9$) or otherwise as stated. The data were then summarized and plotted as an average means ± SE (standard errors). The significance of activity changes was calculated by a 2-tailed *t*-test analysis. A probability value $p < 0.05$ was accepted as significantly different from the control.

Reverse transcriptase-PCR (RT-PCR) analysis. Total RNA was isolated from MLE12 cells. Cultured MLE12 cells prepared for RT-PCR gene analysis were harvested at 80–90% confluence. MLE12 cells, treated with various reagents or transfected with expression plasmid (pCIN-Flag or Flag-PLAGL2) in 24-well plate ($\sim 8 \times 10^5$/well) were harvested for RNA preparation using RNeasy Kit (Qiagene, Valencia, CA). Total RNAs isolated from 3 wells were combined together and then subjected to cDNA synthesis [3].

All of the primers used in the PCR analysis were designed to amplify across exon and intron junctions. For quantitative measurement of GAPDH, SP-C, SP-B, TTF-1, and PLAGL2 transcripts, real-time PCR analysis was employed by incorporating Sybr-green in the amplification reaction and cycled on iCycler (Bio-Rad, Hercules, CA). Primers used for the real-time and RT-PCR analyses were listed in the previous manuscript. The gene transcripts measurement and statistic analysis were performed as previously described [3].

Results

Gene expression in $CoCl_2$ treated lung cells

Exposure to Co^{2+} was known to cause lung disease; however, its concomitant effect on surfactant protein gene expression has never been examined. Here, we evaluated the impact of chemical-induced hypoxia on SP-B and SP-C expression. MLE12 cells were seeded and treated with $CoCl_2$ at a concentration that was not lethal to cells, but with delayed normal cell growth. The concentration, which was applied to cells, was determined by the cell growth curve (Fig. 1A). There was a 2.75-fold increase in the cell counts of 100 μM $CoCl_2$ treated cells versus an 11.7-fold increase in the control sample when compared to the originally seeded cells (Fig. 1A). The chosen concentration for this study was 100 μM and it was similar to that in other report [7]. With this treatment, besides a slower cell growth rate, cell morphology was also changed from spread and adherent in normal control cells (Fig. 1B) to spindle or spherical in treated cells (Fig. 1C). Those cells were not dead according to propidium iodide or trypan blue staining. At a higher concentration of $CoCl_2$ (>200 μM), the decrease of cell numbers indicated the toxicity effects on cell viability.

To examine gene expression in those lung cells under the hypoxia condition, total RNAs were prepared from treated and untreated MLE12 cells and then subjected to quantitative PCR analysis. MLE12 cells express SP-B, SP-C, and TTF-1 type II cell-specific markers. As shown in Fig. 1D, quantitative RT-PCR showed that TTF-1 expression was sensitive to $CoCl_2$ treatment ($58 \pm 7\%$ of the control), but PLAGL2 expression was resistant to the treatment and remained unchanged ($108 \pm 5\%$) (Fig. 1D). Thus, PLAGL2 and TTF-1 clearly have differential responses to $CoCl_2$-induced hypoxia. Regarding SP-B and SP-C expression, there were no significant decreases of their transcripts in treated cells ($172 \pm 23\%$ and $83 \pm 4\%$, respectively). Certainly, the amounts of SP-B and SP-C messages were not parallel to TTF-1 reduction in $CoCl_2$ treated cells.

The SP-C promoter remains active in cells under the hypoxia condition

The mechanism by which SP-B and SP-C transcripts were remained in $CoCl_2$ treated MLE12 cells could be due to continuously activated promoter. To test this possibility, luciferase reporter gene driven by the promoters were employed for examination. With higher PLAGL2 protein level than that in MLE12 cells [3], H441 cells were utilized for the investigation. Similar to the response of