ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Dosage effects of Clara Cell 10 kD (CC10) protein on the human airway epithelial cell line Calu-3 under normoxic and hyperoxic conditions

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ABSTRACT

Recombinant human clara cell 10 kd protein (rhCC10) has been shown to reduce pulmonary inflammation when delivered intratrachealy or intravenously. We evaluated the dosage effects of rhCC10 on human airway epithelial cell line Calu-3 grown at an air-liquid interface culture. Calu-3 cells were treated with rhCC10 protein (0.5, 1.5 and 5 mg/ml) on the apical surface ofcells exposed to normoxia and hyperoxia for 48 hrs. Cell viability studies by trypan blue exclusion indicated no difference in cellular viability between controls and CC10 treated cells. A similar ratio of viable cells (>75%) was observed under both normoxic and hyperoxic conditions and at all concentrations of CC10.After 24 hrs of CC10 treatment, a decreasing trend in transepithelial resistance (TER) values of Calu-3 monolayers under hyperoxic conditions indicating loss of Calu-3 monolayer integrity due to hyperoxic environment. This change was not observed within 24hr of CC10 treatment. The cytotoxicity assay using Lactate dehydrogenase (LDH) activity showed a moderate increase in cellular toxicity with > 1.5 mg/ml of CC10 concentration Therefore, 24 hr of CC10 treatment is the ideal time point to evaluate dosage effects, since viable cells seen under both normoxic and hyperoxic conditions at all concentrations of CC10 treatment. These *in vitro* studies suggest that apical surface treatment of airway epithelia with rhCC10 may exhibit a protective effect under hyperoxic conditions upto 24 hr and prolonged exposure may result in loss of cellular integrity.

Keywords: Clara cell 10 kD, Calu-3, normoxic, hyperoxic

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ISSN: 0975-8585

INTRODUCTION

Clara cells are nonciliated secretory epithelial cells lining the pulmonary airways, distinct from mucous and serous secretory cells in morphology and their secretory products. Clara cell secretory protein (CCSP) is the most abundant secreted protein within airways of the lung.In an exploratory study of the proteins in lung lavage, Singh and Katyal identified a 10 kD protein that is primarily expressed by bronchial Clara cells in rodents and humans [1]. The protein is structurally similar to rabbit uteroglobin and the two proteins appear to be closely related. The protein is referred to in the literature by various names, e.g., uteroglobin, Clara cell secretory protein (CCSP), Clara cell 16 kD protein, Clara cell 10 kD protein (CC10), human protein 1, urine protein 1, and polychlorinated biphenyl-binding protein [1].Despite extensive characterization of the structure of CC10, there is hardly information on its physiological role in the lung. Clara cells are increasingly recognized as major respiratory tract protectors. They serve as stem cells in bronchial epithelial repair, have high xenobiotic transformation capacity, and, through the Clara cell 10-kD protein, counter regulate inflammation [1, 2].

Human airway epithelial cell line Calu-3, which expresses high levels of functional cystic fibrosis transmembrane conductance regulator (CFTR) has been extensively used in understanding the CFTR-mediated ion transport defect in cystic fibrosis (CF) [3,4,5]. It has also been shown that the antibacterial activity of apical surface fluid (ASF) washings from Calu-3 cells grown at an air-liquid interface has a number of similarities to previously characterized antibacterial activity of ASF from primary cultures of airway cells [5]. The regulatory mechanisms of airway fluid secretion are difficult to study *in vivo*, because of its small volume. Several model systems have been used to functionally evaluate fluid secretions and bioelectric properties of airway epithelia [6-9].

The role of CC10, immuno-regulatory and anti-inflammatory peptide [1, 2, 10], on Calu-3 cells exposed to normoxic and hyperoxic conditions will be tested by growing Calu-3 cells at a air-liquid interface. Our hypothesis is that CC10 should provide protection to the Calu-3 cell line when exposed to a hyperoxic insult. We have chosen Calu-3 cells, a well-characterized airway cell line that forms high resistance monolayers when grown on permeable supports (transwell polycarbonate porous inserts), which allows for culture at an air-liquid interface for 23 days. Under these conditions Calu-3 cells form a well-differentiated tight monolayer which generates significant TER, a characteristic of bronchiole epithelium *in vivo*. At air-liquid interface culture, Calu-3 cells develop a TER value >300 ohm.cm^2 in about 11 days, indicating formation of tight junctions in monolayers. In this study we evaluated the protective role of rhCC10 protein on Calu-3 cells under hyperoxic insult.

MATERIALS AND METHODS

Methods

At an air-liquid interface culture, Calu-3 monolayers were grown by exposing apical surface to air and cells were fed basolateraly with cell culture medium. Calu-3 monolayers will

be apically exposed to normoxic ($FiCO_2$ = 5 %, balance air) or hyperoxic (FiO_2 = 95 %, balance CO_2) gas using modular incubator chamber (MIC-101, billups-rothenberg,Inc, delmar,CA) incubated at 37^0C. We believe this model utilizing Calu-3 grown at air-liquid interface simulates the mechanisms of lung injury recognized in clinical practice, similar in dose ratio to intratracheal applications in animal models.

Calu-3 cell culture

Calu-3 cells were cultured at 37° C and 5 % CO_2 in 1:1 mixture of Dulbecco's modified eagle's medium/Ham's F-12 (DMEM/F12) that was supplemented with 15 % fetal calf serum (FCS), 500 U/ml penicillin and 50 µg/ml streptomycin. Cells were maintained in 75-cm^2 tissue culture flasks and split when 80 to 90% of confluency was reached. For growing Calu-3 in transwell inserts (0.4 µm pore size, 12 mm diameter, clear polyester membrane), the cells were plated at 2 x10^6 cells/cm^2 onto transwell inserts that had been precoated with human placental collagen. The medium was added to both the apical and basolateral compartments. An air-liquid interface culture was initiated by removing medium from the apical compartment on the second day after plating cells and the apical compartment containing cells was exposed to air. The medium (0.5ml) in the basolateral compartment was changed every 2-3 days. After approximately 11 days of culturing at air-liquid interface, the TER of the monolayers was measured by adding medium (0.5ml) to the apical side and then using chopstick electrodes and an epithelial volt-ohm meter (World Precision Instruments, Sarasota, FL). Confluent monolayers with a TER value of greater than 300 ohm·cm^2 were apically treated with rhCC10 protein by adding CC10 on top of the Calu-3 monolayers and air-liquid interface culture continued. Sham treated monolayers were used as controls.All cell culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA), with the exception of human placental collagen (Cohesion Technologies, Inc., Palo Alto, CA). Polyester membrane transwell-clear inserts for air-liquid interface culture was obtained from Fisher Scientific.

Culturing Calu-3 cells under normoxic and hyperoxic conditions

Calu-3 cells were exposed at normoxic ($FiCO_2$ = 5 %, balance air) or hyperoxic (FiO_2 = 95 %, balance CO_2) conditions using a modular incubator chamber (MIC-101, Billups-rothenberg, Inc, delmar, CA) at 37°C. For obtaining normoxic conditions, a transwell plate with cells and lid opened were kept inside MIC chamber, both culture plate and MIC chamber were maintained opened in CO_2 incubator (Forma Scientific, Mariotta, OH) for 30 minutes as shown in opened position of chamber (Figure 1B). The lid of transwell tissue culture plate was also kept opened in the same way as MIC chamber. After 30 minutes of exposure, the lid was immediately placed on top of the transwell plate, and the upper and lower chambers of MIC-101 chamber were tightly closed. For obtaining hyperoxic conditions, a transwell plate with cells and lid on top was kept inside the MIC-101 chamber, lids were tightly closed and the chamber was purged with FiO_2 = 95 %, balance CO_2 (Air gas, Radnor, PA) by opening both inlet and outlet ports. The purging of hyperoxic gas was performed at a flow rate of 20 liters/min for 3 minutes, after which the gas source was disconnected, and the chamber was sealed by closing plastic clamps. In the hyperoxic condition, the presence of a constant oxygen environment inside the

experimental chambers was monitored by means of a MiniOX (oxymeter) maintained inside the chamber during the study. Both normoxic and hyperoxic experimental chambers were kept at 37°C within CO_2 incubator. The chambers were humidified by placing a petri-dish containing 20ml of sterile water inside. For both conditions, the gas in the chamber was changed every 24 hr by opening and closing the chambers.

Calu-3 cell treatment with CC10

The effect of CC10 under hyperoxic stress will be tested for 48 hrs using 4 different concentrations of rhCC10 protein (0, 0.5, 1.5 & 5.0 mg/ml), by adding protein solution to apical surface of monolayers. Recombinant human CC10 protein was obtained as gift from Dr.AprileL.Pilon. To assess the Calu-3 monolayer integrity, TER measurement was done after 24 hr and 48 hr of rhCC10 treatment. After rhCC10 treatment, ASF washings were collected from Calu-3 cells. Cytospin preparations of Calu-3 cells were made using cell suspensions obtained by harvesting the cells from transwell filters by trypsinization.Cytospin preparations were stained with hemotoxylin-eosin and examined by light microscopy.assay was tested by Trypan blue exclusion and cytotoxicity was tested by Lactate dehydrogenase assay (LDH). Data analyses will be conducted to differentiate CC10 treated and non-treated Calu-3 cells under both normoxic and hyperoxic conditions.

RESULTS

Calu-3 cells were grown at an air-liquid interface, a monolayer with a tight junction forms in about 11 days as demonstrated by the development of a TER >300 ohm·cm^2. After the development of tight junction as monitored by TER values, cells were treated with various concentrations of CC10 under normoxic and hyperoxic conditions for 24 and 48 hrs and the results are compared between them.The measurement of TER directly indicates Calu-3 monolayer integrity under normoxic and hyperoxic conditions. Our results showed that after 24 hrs of CC10 treatment, a decreasing trend in TER values of Calu-3 monolayers under hyperoxic conditions, which indicated loss of monolayer integrity due to hyperoxia induced cellular injury (Figure 1). This change was not observed within 24hr of CC10 treatment.

The presence of viable cells was seen at all concentrations of CC10 as well as under normoxic and hyperoxic conditions. No difference in cell viability was observed intrypan blue exclusion studies (Figure 2), and > 75% of viable cells was seen at all concentrations of rhCC10upto 48 hrs. Similarly, no significant difference in cell viability was observed between controls and CC10 treated cells. The cytotoxicity assay using LDH activity showed a moderate increase in cellular toxicity with > 1.5 mg/ml of CC10 concentration (Figure 3). At 5 mg/ml of CC10, an increased number of cells with cytomorphological changes were observed in cells treated with CC10 for 48 hrs under both normoxic and hyperoxic conditions as examined with cytospin preparations (Figure 4). Such cytomorphological changes were characterized by disrupted cell morphology with enlarged and diffused cytoplasm. Though LDH assay indicated cytotoxicity with high doses of CC10, TER values and cell viability by Trypan blue exclusion studies indicated no loss of viability. Therefore, higher doses of CC10 (>1.5 mg/ml) may not be

ISSN: 0975-8585

suitable concentration for further studies.The presence of viable cells was seen under both normoxic and hyperoxic conditions at all concentrations of CC10 treatment indicated that CC10 treatment resulted in no significant difference in cell viability and monolayer integrity between normoxic and hyperoxic conditions indicating protective role of CC10 under hyperoxic conditions.

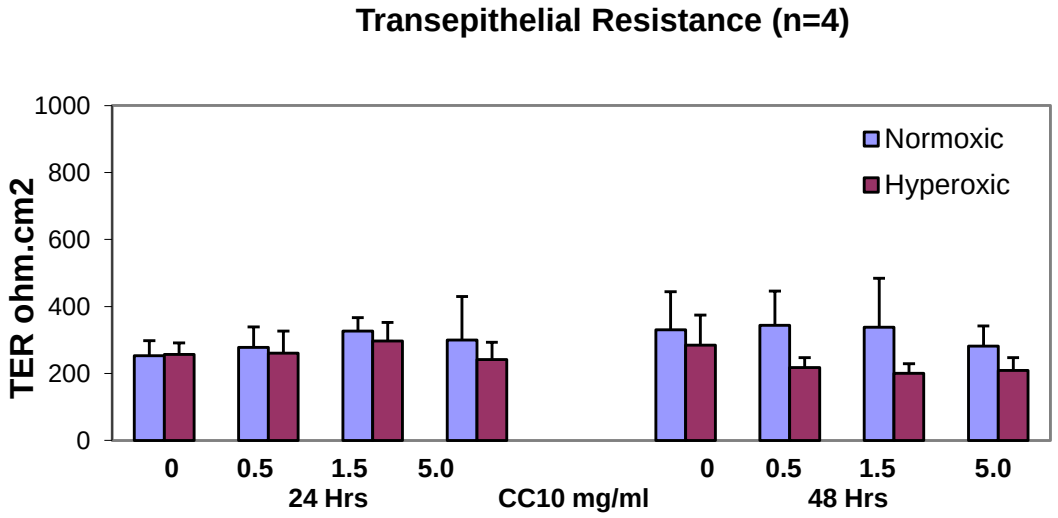


Figure 1 :Transepithelial Resistance (TER) was measured using epithelial volt-ohm meter after treating Calu-3 cells with CC10 protein (n = 4). TER values were compared between normoxic and hyperoxic conditions. A decreasing trend in TER value was observed after 24 hrs under Hyperoxic conditions

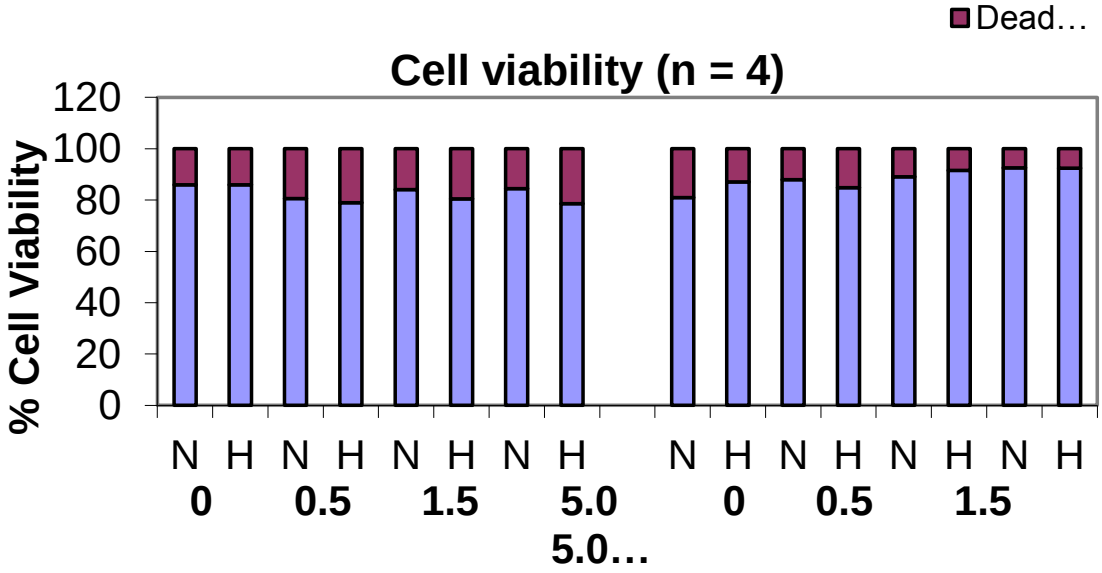


Figure 2 :Examination of cell viability by trypan blue exclusion studies after treating Calu-3 cells with rhCC10 protein (n = 4). % Cell viability was calculated by measuring live and dead cells under light microscope using cell counting Chamber. > 75 % of viable cells were observed under all conditions including hyperoxic condition and there was no significant change in cell viability upto 48 hrs. N = Normoxic& H = Hyperoxic

ISSN: 0975-8585

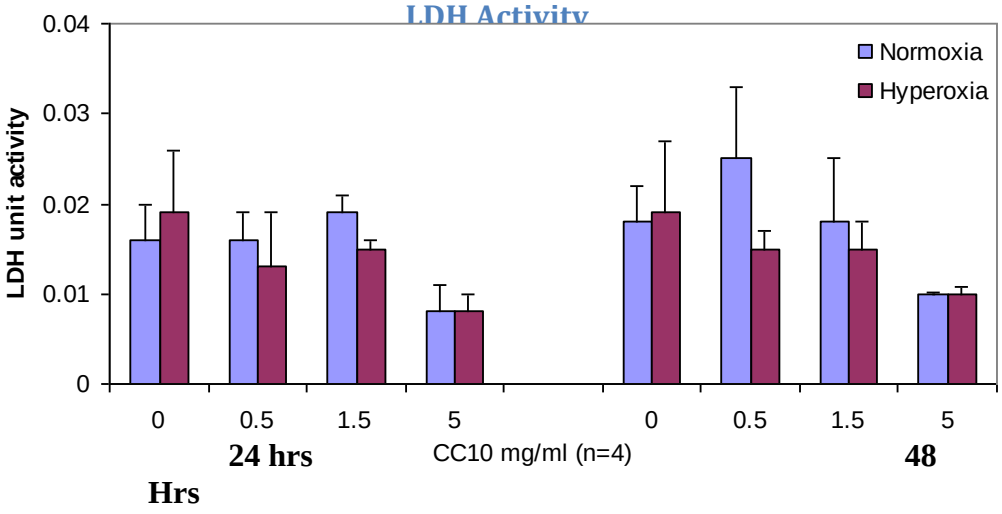
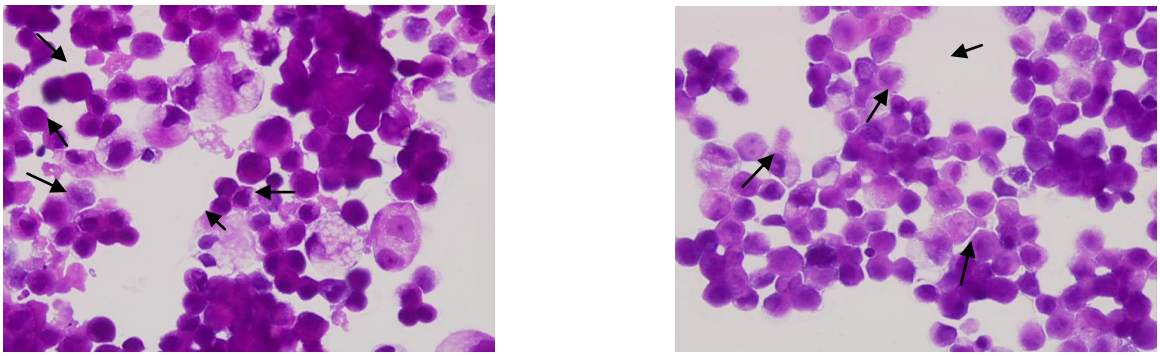


Figure 3 : Calu-3 cells treated with rhCC10 protein were tested for cytotoxicity by Lactate dehydrogenase assay (LDH). The results were compared between normoxic and hyperoxic conditions after 24 hr and 48 hr. Cytotoxicity with CC10 was observed above 1.5 mg/ml of CC10 concentration.



48 Normoxiacytospins– CC10 5mg/ml **48 Hyperoxiacytospins – CC10 5mg/ml**

Figure 4 : Microscopic examination of Calu-3 cytospins. Cytospin preparations were stained with hematoxylin and eosin (n=4 for each condition). All cytospins were examined by light microscopy at 40X magnification. Arrow marks indicate cytomorphological changes observed after CC10 treatment.

DISCUSSION

In the present study we evaluated the effect of rh CC10 when airway epithelial cells are grown at air-liquid interface culture. These studies employed Calu-3 cells, a well-characterized airway cell line that forms high resistance monolayers when grown on permeable supports, allowing for culture at an air-liquid interface for 23 days [11, 12, 13]. Under these conditions Calu-3 cells form a well-differentiated tight monolayer which generates a significant TER, a characteristic of bronchiolar epithelium *in vivo*. The Calu-3 cell line has previously been employed as a model to screen possible drug candidates and formulations to be delivered to the respiratory epithelium [11-14]. Calu-3 cells grown at air-liquid interface simulates

pulmonary airway epithelial cells *in vivo,* wherein cells are under constant exposure to environmental air, and taking advantage of Calu-3 cells growing at air-liquid interface [16, 17, 18], we were able to test the dosage effects of CC10 protein. Under such conditions, we tested the monolayer integrity, cellular viability and cytomorphological changes under hyperoxic conditions and results were compared with normoxic conditions. Such a study is highly beneficial in testing physiological and pharmacological effects when cells are grown in direct contact with air.

Previously Calu-3 cells have been used as a model to examine transport and metabolism of many pharmacological compounds [14-17]. In earlier models of continuous positive airway pressure, in which Calu-3 monolayers were exposed to normoxia (control), hyperoxia, pressure (+20 cm H_2O), or a combination of hyperoxia and positive pressure, a group effect for cell viability revealed that hyperoxia was less detrimental than pressure, but both insults in combination are more detrimental than either independently [17].

The Calu-3 cell culture model developed in the present study will be helpful to investigate potential therapeutic benefit of CC10 protein. In previous studies we demonstrated that hyperoxic conditions induced cellular injuries in Calu-3 grown at Air-liquid interface. In the present study our results showed viable cells seen under both normoxic and hyperoxic conditions at all concentrations of CC10 treatment indicated that CC10 treatment resulted in no significant difference in cell viability between normoxic and hyperoxic conditions. Therefore, our*in vitro* studies suggest that apical surface treatment of airway epithelia with rhCC10 may exhibit a protective effect under hyperoxic conditions by maintaining the integrity of epithelial monolayers, however prolonged duration (>24hr) may not be beneficial. Thus, *in vivo*, intratracheal delivery may be the most promising mode of rhCC10 administration for the clinical management of respiratory inflammation.

ACKNOWLEDGEMENTS

The encouragement and support from BharathUniversity, Chennai.India is gratefully acknowledged. The authors thank Nemours Research Foundation, Delaware.USA for their support and funding. The authors also thank Dr.Aprile L. Pilon for providing CC10 protein.

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