Toxicon 54 (2009) 184–191

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Translocation of ricin across polarized human bronchial epithelial cells

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ARTICLE INFO

Article history:
Received 12 December 2008
Received in revised form 30 March 2009
Accepted 1 April 2009
Available online 15 April 2009

Keywords:
Ricin
Transcytosis
Translocation
Bronchial epithelial cells
Inhalational toxicity

ABSTRACT

Due to widespread availability, toxicity, and potential for use as a bioterrorism agent, ricin is classified as a category B select agent. While ricin can be internalized by a number of routes, inhalation is particularly problematic. The resulting damage leads to irreversible pulmonary edema and death. Our study describes a model system developed to investigate the effects of ricin on respiratory epithelium. Human bronchial epithelial (HBE) cells were cultured on collagen IV-coated inserts until polarized epithelial cell monolayers developed. Ricin was added to the apical or basal medium and damage to the cell monolayer was then assessed. Within a few hours after exposure, the cell monolayer was permeable to paracellular passage of the toxin. A mouse anti-ricin antibody neutralized ricin and prevented cellular damage as long as the antibody was present before the addition of toxin. These studies suggested that effective therapeutic agents or antibodies neutralizing ricin biological activity must be present at the apical surface of epithelial cells. The *in vitro* system developed here provides a method by which to screen potential therapeutics for protecting lung epithelial cells against ricin intoxication.

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1. Introduction

Native to tropical east Africa, castor bean plants (*Ricinus communis*) are commercially cultivated in many areas of the world for their valuable oil used in numerous products, such as soaps, cosmetics, medicines, motor oil, and nylon (Olsnes, 2004). Castor bean plants, grown for ornamental purposes, have also become weeds in many temperate areas of the world. In addition to the oil, castor beans contain ricin, a highly toxic protein (Bigalke and Rummel, 2005). A number of factors, such as widespread availability, ease of extraction, high toxicity, stability, and lack of specific antidotes, contribute to concerns about ricin being used as a biological threat agent. The ease with which aerosol dispersal of ricin could affect large populations justifies the United States Centers for Disease Control (CDC) placement of ricin as a category B agent (Khan et al., 2000).

Ricinus communis agglutinin II (ricin) belongs to the type 2 ribosome-inactivating protein family consisting of enzymatic and cell binding subunits linked by a disulfide bond. The enzymatic subunit, ricin A chain (RTA), excises a specific adenine residue in mammalian 28S rRNAs, the removal of which prevents elongation factor 2 from binding amino acids into a polypeptide chain, thereby inhibiting protein synthesis and causing cell death (Endo and Tsurugi, 1988; Nilsson and Nygard, 1986). By binding to galactoside residues present on cell-surface glycoproteins, the cell binding subunit, ricin B chain (RTB), enables ricin to be endocytosed. Once in the cell, the two chains separate and RTA is translocated into the cytosol, gaining access to ribosomes (Olsnes, 2004). Because ricin binds to many cell-surface receptors, almost all mammalian cell types are susceptible to ricin intoxication.

The symptoms and severity of ricin intoxication depend on the delivery route. Aerosolized ricin is considered a serious threat because it induces severe lung damage (Audi et al., 2005). Depending upon the dose, symptoms may include pulmonary edema and hypoxic respiratory

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0041-0101/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.toxicon.2009.04.003

failure, with fever, cough, dyspnea, nausea, chest tightness, cyanosis, hypotension, and circulatory collapse (Audi et al., 2005; Bigalke and Rummel, 2005). Because there is no antidote for ricin intoxication, treatment of victims is strictly palliative. Death usually ensues within 3–5 days, although patients who survive this critical initial period have a good prognosis (Bigalke and Rummel, 2005).

In order to develop effective drug therapies for ricin intoxication, identifying routes of intoxication becomes important, particularly since the toxin binds to and then kills most human cells. In the case of aerosolization, the first cells encountered are in the respiratory epithelium that forms a protective barrier over the underlying tissues. Because a lethal dose of ricin instilled via the pulmonary system may lead to systemic distribution with damage to the kidneys and other organs, characterizing how it crosses the epithelial cell barrier becomes a critical issue (Wong et al., 2007).

One method by which ricin might cross the epithelial cell barrier is by transcytosis, a process by which proteins cross from one side of the cell to the other as membrane-bound cargo (Tuma and Hubbard, 2003). Ricin has been shown to cross polarized Madin–Darby canine kidney (MDCK) and the human colon adenocarcinoma epithelial (Caco-2) cell monolayers via transcytosis, suggesting that this process may also be used by the toxin to cross lung epithelia (Brandli et al., 1990; Jackman et al., 1994). Therefore, an *in vitro* model was developed to determine if ricin uses transcytosis to cross the lung epithelial cell barrier. Unlike previous investigations with kidney and intestinal epithelial cells, our studies indicated that transcytosis did not play an important role in moving ricin from the lung into the vascular system. Rather, damage to the tight gap junctions permitted toxin to move around the cells, thus gaining entry by paracellular diffusion.

2. Materials and methods

2.1. Materials

Ricinus communis agglutinin II (ricin) was purchased from Vector Laboratories, Burlingame, CA. Mouse anti-deglycosylated ricin toxin A chain antibody (anti-dgRTA IgG) was prepared by PerImmune, Inc. (Rockville, MD) under contract for United States Army Medical Research Institute of Infectious Diseases, Frederick, MD (Lindsey et al., 2007). Alexa Fluor 680 antibody kits, a 10,000 MW dextran conjugated to Alexa 680 (dextran-680), rabbit anti-ZO-1 IgG, goat anti-rabbit IgG conjugated to Alexa 488, Texas Red phalloidin, Hoechst 33342 dye, polyacrylamide gel electrophoresis (PAGE) supplies, Earle's minimal essential medium (EMEM), RPMI-1640 medium, Hank's balanced salt solution (HBSS), L-glutamine (200 mM), non-essential amino acid (10 mM) solution, and penicillin–streptomycin liquid (10,000 units penicillin; 10,000 μg streptomycin) were obtained from Invitrogen, Carlsbad, CA. Bronchial epithelial growth medium (BEGM) and trypsin ReagentPak were purchased from Lonza (Walkersville, MD). Fetal bovine serum (FBS) was purchased from Hyclone, Logan, UT, and heat inactivated (56 °C, 30 min) before use. Cell culture plates and flasks were obtained from Corning Life Sciences, Corning, NY. BD Biocoat collagen type IV-coated 24-well plate inserts (1.0-μm pores) were purchased from BD Biosciences, Bedford, MA, while the Nunc Anopore membranes (0.02-μm pores) were from Nalge Nunc, Rochester, NY. The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (CellTiter 96AQ) was purchased from Promega Corporation, Madison, WI. A bicinchoninic acid-based (BCA) kit to measure protein concentrations was purchased from Thermo Fisher Scientific, Inc., Rockford, IL. Immunoglobulin and protease free bovine serum albumin (BSA) was purchased from Jackson ImmunoResearch, West Grove, PA and chemicals from Sigma Aldrich Chemicals, St. Louis, MO. Molecular weight markers for PAGE and Coomassie Blue protein stains were purchased from LI-COR, Inc., Lincoln, NE.

2.2. Labeling ricin with Alexa 680

To follow ricin translocation across epithelial cell monolayers, ricin was conjugated to a dye that fluoresces in the near infrared (NIR) range (Rushing et al., 2007). Ricin (1.5 mg) was suspended in 0.5 ml of phosphate-buffered saline (PBS), pH 7.2, and labeled with the Alexa Fluor 680 NIR dye according to the manufacturer's instructions. Final concentrations of labeled proteins were measured by the BCA protein assay. SDS-PAGE gel electrophoresis conducted under non-reducing conditions revealed a single labeled band of approximately 60 kDa when scanned in the 700 nm channel of the Odyssey imaging system (LI-COR, Inc.). The gel, subsequently stained with Coomassie Blue protein stain, showed that both unlabeled and labeled ricin (1 μg/ml) had a 60 kDa band of similar density. When assessed using Jurkat cells, both the NIR-labeled ricin (ricin-680) and the unlabeled ricin standard exhibited similar cytotoxicity, indicating that labeling had no effect upon biological activity (data not shown).

2.3. Cell culture

The 16HBE14o− human bronchial epithelial cell line (HBE) was obtained from Dr D.C. Gruenert, California Pacific Medical Center Research Institute, San Francisco, CA. HBE cells were maintained in EMEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 0.1 mM non-essential amino acid solution, penicillin (50 units/ml), and streptomycin (50 μg/ml). When the monolayer reached approximately 80% confluency, cells were trypsinized using the ReagentPak, and then subcultured in 175 cm^2 tissue flasks with cells for experiments between passages 4 and 16. The Jurkat human T lymphocyte leukemia cell line (American Type Culture Collection, Manassas, VA) was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FBS (RPMI-FBS), and passed twice weekly. All cells were maintained in a 37 °C incubator with 5% CO_2.

2.4. Ricin cytotoxicity assay

Ricin biological activity was determined using a Jurkat cell viability assay in which Jurkat cells were resuspended in RPMI-FBS at a density of 2×10^6 cells/ml and transferred to a 96-well plate (100 μl/well). Samples (50 μl) were then