EFFECTS OF RECOMBINANT SURFACTANT PROTEIN D (rSP-D) ON ENDOTOXIN INDUCED CHANGES IN THE METABOLISM OF ENTEROCYTE LIKE CACO-2 CELLS

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Abstract

Surfactant protein D (SP-D), a collagenous C-type lectin, plays an important role in the regulation of the innate immune response in lungs. Expression of SP-D has also been reported at many extrapulmonary sites including intestine. The objective of this study was to examine the effects of endotoxin on enterocyte like Caco-2 cell metabolism and to investigate whether recombinant SP-D (rSP-D) might have a protective response against possible endotoxin induced metabolic changes in Caco-2 cells. Our results demonstrate that Lipopolysaccharide (LPS) alone inhibits RNA and glyco(protein) synthesis while in combination with rSP-D, no alteration of cellular metabolism is observed. Furthermore, LPS alone causes cell death while the addition of SP-D gives protection against these toxic effects of LPS. Since intestinal epithelium cells are constantly in contact with bacterial endotoxins, we speculate that SP-D is part of a natural protection mechanism in the intestinal tract.

INTRODUCTION

The mucosal surface of the gastrointestinal tract (GIT) is covered with a one cell layer, the mucosal epithelium. Intestinal epithelium is constantly exposed to gram negative bacteria, which are able to directly deposit their toxic and proinflammatory constituents such as LPS at the intestinal epithelial apical surface. LPS may then be internalized, recycled, stored, or transcytosed from the apical to the basolateral pole of the intestinal epithelium (1). LPS is a potent toxin that elicits several immediate proinflammatory responses in mammalian cells. Despite the density of these bacteria and their toxins, the intestinal epithelium does not activate proinflammatory responses to these organisms. Both innate and acquired immune systems protect the GIT against microbial endotoxins.

Surfactant protein D (SP-D) is a collagenous glycoprotein which belongs to a family of proteins implicated in innate immunity termed as collectins (2). Other members of the collectin family are Surfactant protein A (SP-A), mannose binding lectin (MBL) and bovine conglutinin. Collectins share the same basic structural properties: they are multimers consisting of a short cysteine-rich, N-terminal cross-linking domain, followed by a collagen domain, an α-helical neck domain and a C-type lectin (calcium-dependent) domain, or a carbohydrate recognition domain (CRD) (3). Alveolar type II cells and Clara cells (uncilliated bronchial epithelium) of the lung are the major sites of synthesis of surfactant proteins (4). SP-D has been extensively studied in lungs and it plays an important role in the host defense and inflammatory processes in lungs (5). SP-D can recognize a spectrum of pathogens and can interact with a number of viruses, bacteria and fungi (6). Both surfactant proteins SP-A and SP-D are reported to be synthesized in different species by a

variety of extra-pulmonary tissues including middle ear duct, oesophagus, stomach, intestine and peritoneal cavity mesentery (7). In a recent study expression of SP-D has been shown in human gastric antral mucosa at both the mRNA and the protein levels. In the same study, it has been demonstrated that the gastric epithelium is capable of high levels of SP-D expression in the context of *H. pylori* infection (8). Expression of this protein at this site suggests that SP-D may play a role in host defense of the GIT. SP-D binds directly to LPS on the surface of gram-negative bacteria via the carbohydrate recognition domain. This process may result in the aggregation of microorganisms followed by enhanced phagocytosis by neutrophils and macrophages (9).

Caco-2 cell culture displaying enterocyte like differentiation is a suitable model for studying proliferation and differentiation mechanisms of intestinal epithelial cells at the cellular level. Differentiated cells exhibit structural and functional properties of the small intestinal villous enterocytes (10).

The objective of this study was to examine the effects of endotoxin on enterocyte like Caco-2 cell metabolism and to determine whether rSP-D might have a protective response against possible endotoxin induced metabolic changes in Caco-2 cells.

MATERIALS AND METHODS

Expression and purification of recombinant SP D (rSP-D):

Recombinant homotrimeric fragment composed of collagen region, α-helical coiled-coil neck region and CRDs of human SP-D was prepared in *E. coli* BL21 (DE3) as described by Kishore *et al* (11). rSP-D preparation was analyzed for bacterial endotoxin contamination with a *Limulus* amebocyte lysate (LAL) assay (BioWhittaker, Walkersville, MD). The amount of endotoxin present in the rSP-D preparation was found to be 331 pg of endotoxin per μg of rSP-D.

Lipopolysaccharide (LPS)

LPS (*E. coli* O55:B5) used in this study was taken from the LAL kit.

Caco-2 cell culture and incorporation of radioactive precursors after incubation with rSP-D and LPS:

Caco-2 cells were cultured for 19 days to achieve fully differentiated cell populations. This study encompassed cell passage numbers 40-50. On day 19 of culture, different concentrations of rSP-D and endotoxin were added to the cells in low glucose (5.54mM glucose) and serum free medium. Radioactive precursors ^{14}C-thymidine (0.02 μCi), ^{3}H-glucosamine (0.8 μCi) and ^{14}C-uridine (0.02 μCi), ^{35}S-methionine (0.1 μCi) (Amersham, Nederland BV) were added to the monolayer and double labelling incubation was performed according to Hendriks *et al.* (12). The incorporated radioactivity was calculated as counts per minute per well and was expressed as relative incorporation.

RESULTS AND DISCUSSION

The results of relative incorporation of precursors after incubation with rSP-D and LPS are represented in the fig. 1 and 2 respectively. Relative incorporation of ^{14}C-uridine, ^{35}S-methionine and ^{3}H-glucosamine decreases significantly with increasing concentration of endotoxin, where as there is little or no change in the relative incorporation of ^{14}C-thymidine. None of the

concentrations of rSP-D in the range of 8ng/ml to 1000ng/ml seems to produce significant differences in the relative incorporation of radioactive precursors.

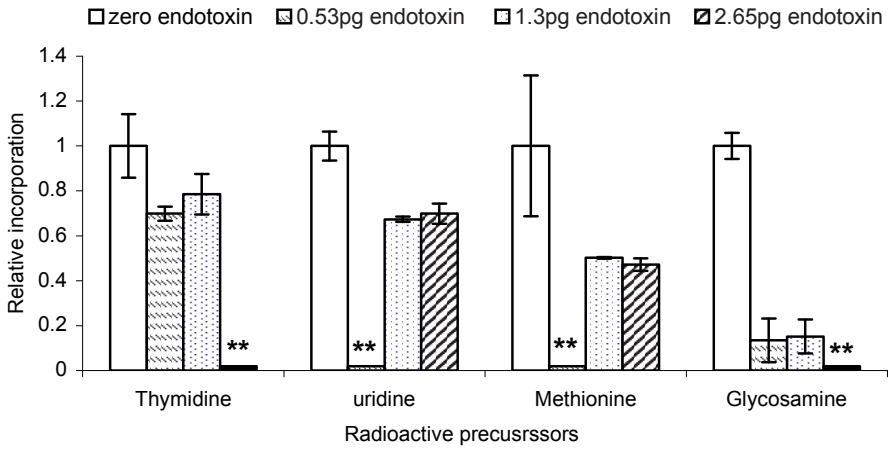

Figure 1. *Relative incorporation of ^{14}C-thymidine, ^{14}C-uridine, ^{35}S-methionine and ^{3}H-glucosamine (precursors for DNA, RNA, glycoprotein and protein synthesis) by differentiated Caco-2 cells following incubation with endotoxin. Data are represented as mean relative incorporation ± SEM. ** No value because of cell death.*

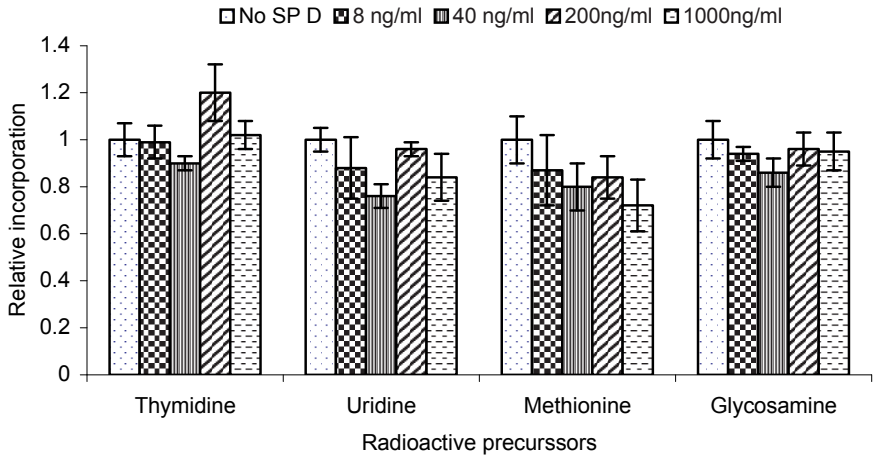

Figure 2. *Relative incorporation of ^{14}C-thymidine, ^{14}C-uridine, ^{35}S-methionine and ^{3}H-glucosamine (precursors for DNA, RNA, glycoprotein and protein synthesis) by differentiated Caco-2 cells following incubation with rSP-D. Data are represented as mean relative incorporation ± SEM.*

Premixing of rSP-D with the LPS of *E. coli* strains O55: B5 for one hour and subsequent incubation of Caco-2 cells indicate that rSP-D increases the cell survival and decreases the inhibitory effects induced by this LPS at higher concentrations (table 1).

Table 1. *Effect of one hour premixing of rSP-D and LPS on Caco-2 cell survival and incorporation of radioactive precursors.*

Conc. (per ml) of pre mixed rSP- D and LPS	Cell Survival (wells)	Average Counts per minute per well*			
		Thymidine	Uridine	Methionine	Glucosamine
1 pg LPS	0/6	-	-	-	-
1 pg LPS+10 ng rSP-D	0/6	-	-	-	-
1 pg LPS+100 ng rSP-D	2/6	663	1565	130	45
1 pg LPS+1000 ng rSP-D	5/6	876	2737	222	44

* Average counts shown were calculated from the wells having survived cells.

In this study we used Caco-2 cells to study the effects of recombinant surfactant protein D and endotoxin on DNA, RNA and (glyco)protein synthesis.

The intestinal epithelium serves as a critical barrier to lumenal bacteria and food antigens. Intestinal epithelial cells are active participants in the intestinal innate immune response. A broad spectrum of peptides has been described to play an essential role in regulating the intestinal immune system to balance the host mucosal defense system, tolerance for resident colonic micro-organisms and repair of intestinal epithelial injury (13). Endotoxin, the bacterial Lipopolysaccharide (LPS) is a characteristic outer membrane entity of gram negative bacteria and a potent inducer of inflammatory responses. LPS is present in massive amounts in the intestine as liberated by the commensal gram negative bacteria. To maintain the tolerance to gram negative bacteria, the normal intestinal epithelium should be unresponsive or hyporesponsive to bacterial LPS. There is not much known about the effect of endotoxin on Caco-2 cells. The results of our study show that LPS decreases RNA and (glyco) protein synthesis in Caco-2 cells.

In lungs LPS can induce lung injury leading to acute respiratory distress syndrome (ARDS). Surfactant replacement has been shown to improve the pulmonary function in ARDS, which displays the host defense capacities of the surfactant other than surface tension lowering activity (14). In lungs, it has been shown by Kuan *et al* (9) that SP-D interacts with LPS of various phenotypes. There is not much literature available for the functional significance of SP-D in the gastro intestinal tract. SP-D is related to the collectin family of proteins, which are components of the innate immune system. The major role of SP-D seems to be recognition of foreign carbohydrate structures expressed on the cell walls of microorganisms (6). SP-D may therefore be one of the first lines of defense in the gastric mucosa. Murray *et al* (8) have demonstrated the expression of SP-D in human gastric antral mucosa at both the mRNA and the protein levels. This study also showed that gastric epithelium is capable of high levels of SP-D expression in the context of *H. pylori* infection. Thus SP-D may play a role in gut host defense. To determine whether surfactant protein D could be involved in the protection from LPS induced inhibition of cellular metabolism, we examined the DNA, RNA and (glyco) protein synthesis in Caco-2 cells after incubation with rSP-D and LPS. LPS was present in the preparation at 331pg/microgram of rSP-D, which is high compared to the concentration of LPS, which produces negative effects on Caco-2 cell metabolism. The results on Caco-2 cell metabolism indicate that rSP-D may bind to LPS and

thus prevents the binding of LPS to intestinal epithelium. In the *in vivo* situation SP-D may contribute to the inactivation of soluble LPS released by gram negative bacteria in the gut.

We report a protective effect of rSP-D on the deleterious effects of *E. coli* (O55:B5) LPS in terms of less cell death and less decrease in the cell metabolism with increasing SP-D concentrations. Future studies are focused on the effects of LPS from other *E. coli* including BL21 (DE3), although the active domain of the patho-physiological effect of LPS is very conserved in *E. coli*. We also plan to investigate the influence of SP-D on pathogen adhesion and invasion mechanism.

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