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Delivery of TEM β-lactamase by gene-transformed *Lactococcus lactis* subsp. *lactis* through cervical cell monolayer

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Abstract

Lactococcus lactis subsp. *lactis* transformed with Plasmid ss80 (encoding the production and secretion of TEM β-lactamase) was used for the delivery of β-lactamase through the C-33A (cervix cell) monolayer. The viability of the cell monolayers co-cultured with *L. lactis* was examined by the trypan blue exclusion method. The integrity of the monolayers was monitored by measuring the transport of mannitol and propranolol as well as the transepithelial electrical resistance. The transport rate of β-lactamase through C-33A monolayer was increased by four- and nine-folds ($p < 0.05$) at the first hour by the transformed *L. lactis* compared to the free solution with or without presence of the untransformed *L. lactis*, respectively. This increase was gradually diminished after the 1st hour: it became 30 and 50% ($p < 0.05$) at 10 h. The presence of the untransformed *L. lactis* with free solution delivery also increased the transport rate by 100% at 1 h ($p < 0.05$) and 15% at 10 h ($p > 0.05$). The increase in transport rate by the transformed *L. lactis* is most probably due to the concentrate of β-lactamase on C-33A monolayer. When co-cultured with the *L. lactis*, the C-33A cell viability and the monolayer TEER remained steady for 10 h. The presence of *L. lactis* did not change the transport of propranolol and mannitol through the monolayers. In conclusion, the transformed *L. lactis* significantly ($p < 0.05$) increased the transport of β-lactamase through the cervical monolayers, indicating probiotic bacteria delivery may be a promising approach for protein delivery through the vagina.

Keywords: C-33A; *Lactococcus lactis*; TEM β-lactamase; Protein delivery; Normal flora; Bioavailability; Vaginal delivery

1. Introduction

Proteins are generally water soluble and cannot be delivered by non-invasive routes; they are usually delivered by injection. The frequent injection of these drugs causes pain, and inconvenience for the patients. There is a need for a non-invasive delivery system for these protein drugs. In the past two decades there have been enormous efforts in developing a non-invasive delivery system capable of achieving the required systemic blood levels of these protein drugs. However, not much success has been achieved mainly because of the poor absorption and extensive degradation of the protein drugs. To overcome these problems encountered in non-invasive protein drug delivery, one approach might be to utilize the genetically engineered normal flora as the delivery system.

Normal flora consists of the non-pathogenic bacteria that exist in the open tracts of the human body such as intestine, nostril, and vagina. By recombinant DNA technology, the normal flora can be genetically engineered to synthesize and secrete protein drugs. The normal flora has a natural tendency to adhere tightly to the epithelial cell surface of the channels where they normally reside (Tuomola and Salminen, 1998). This adherence provides an advantage for the recombinant bacteria in protein drugs delivery, since the bacteria will directly deliver the protein drugs onto the epithelial cell surface where the absorption will take place. The direct delivery of the protein drugs onto the epithelial surface will minimize the enzymatic and bacterial degradation of the protein drugs, and will also concentrate

Abbreviations: ATCC, American Type Culture Collection; AUC, area under the curve; C-33A, human cervix carcinoma; CPM, counts per minute; cfu, colony forming units; DMEM, Dulbecco's minimal essential media; FBS, fetal bovine serum; *L. lactis*, *Lactococcus lactis*; PBS, phosphate buffer saline; s-DMEM, supplemented DMEM; TEER, transepithelial electrical resistance

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the protein drugs at the absorption surface. Thus, the absorption may be increased. Studies have shown that fibrillae, strands of non-structured material associated with the lactobacillus cell surface, as well as surface adherent molecules enable the bacteria to attach to epithelial cells and other substrata (Tannock, 1992).

Lactic acid bacteria (LAB) are remarkably diverse group of gram-positive bacilli that are ubiquitous components of the normal indigenous flora of humans and animals. LAB have been isolated from the open tracts of the human body such as intestine, nostril, and vagina. LAB bacteria are often used in food processing and food preservation (Pouwels et al., 1998). Administration of viable lactobacillus strains has been described as therapeutic for diarrhea and other intestinal disorders (Sullivan and Nord, 2002), vaginitis (Reid et al., 1990), and urinary tract infections (Velraeds et al., 1996).

Lactococcus lactis, one of the safest LAB (Salminen et al., 1998), was chosen in the present study as a vector for the delivery of β-lactamase, a model protein of 29 kDa, through a model vaginal epithelial monolayer. The expression–secretion cassette (Plasmid ss80) for the host *L. lactis* was made and confirmed for expression and secretion of β-lactamase into the culture medium (Sibakov et al., 1991). Our previous studies showed that the *L. lactis* significantly increased the transportation of β-lactamase through Caco-2 monolayer and almost doubled the transportation rate as compared to solution form (Shao and Kaushal, 2004). Due to the intensive hepatic metabolism (1st-pass effect), most of the drugs show poor oral bioavailability even though the intestinal absorption is good. The vaginal route might be advantageous over the oral route since the former avoids the hepatic 1st-pass effect, and the vaginal epithelium is permeable to a wide range of molecules, like hormones, antimycotics, peptides and proteins, and its large surface area and rich blood supply make it a promising site for drug delivery (Richardson and Illum, 1992). In addition, a prolonged contact of a delivery system with the vaginal mucosa may be achieved more easily than at other absorption sites like the rectal or intestinal mucosa. In post-menopausal women, the reduced epithelial thickness may increase the drug absorption (Furuhjelm et al., 1980). Therefore, following our previous studies on normal flora delivery through Caco-2 monolayers, we have investigated the delivery of β-lactamase by normal flora through a model cervix epithelium—the C-33A monolayer in the current study.

The cervix is the channel between the vagina and uterus. Different types of cervix cell lines like C-33A, HeLa, CaSki, and many more have been used in numerous studies, most of which were cancer-oriented studies. C-33A is derived from the humans and shows epithelial morphology. It is one of the most commonly used cervical cell line and thus has been used for this study also. Drug absorption from these model cervical epithelia may be used to predict the in vivo absorption through vagina.

The main objective of the present study is to investigate the protein delivery efficacy by normal flora through C-33A monolayer in order to estimate the in vivo vagina absorption with β-lactamase as the model protein and *L. lactis* as the model normal flora. The secondary objectives of the present study are to explore the feasibility of using the in vitro cell culture technique to study the vaginal drug delivery by normal flora, and to examine the possible effects of the *L. lactis* on the C-33A monolayer.

2. Materials and methods

2.1. Materials

C-33A, a human cervical cell line, was purchased from ATCC (Rockville; MD, USA). *Lactococcus lactis* subsp. *lactis* (*L. lactis*), transformed with Plasmid ss80 containing the gene of β-lactamase was generously provided by Dr. Soile Tynkleynen (Valio Ltd., Helsinki, Finland). Dulbecco's modified eagle medium (DMEM), 0.25% trypsin with 0.2 g/l EDTA, fetal bovine serum (FBS), sodium pyruvate (11 mg/ml) and non-essential amino acids (100×) were obtained from Hyclone (Logan, UT, USA); β-lactamase and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA); Transwell® inserts, tissue culture treated (0.4 μm pore size, 4.7 cm^2 surface area), and culture flasks were purchased from Costar Corporation (Cambridge, MA, USA); Bacto M17 broth and Bacto agar were purchased from Becton Dickinson (Sparks, MD, USA). D-mannitol-[1-^{3}H(N)] and DL-propranolol-[4-^{3}H] hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Culture of the C-33A cells

The C-33A cells were cultured in flasks in s-DMEM, DMEM supplemented with 10% (v/v) FBS, 100 I.U./ml penicillin–100 μg/ml streptomycin, sodium pyruvate (0.11 mg/ml), and 1% (v/v) non-essential amino acids at 37 °C in a 5% CO_2–95% air with high humidity. Upon confluence, the cells were harvested by the treatment of 0.25% trypsin with 0.2 g/l EDTA, resuspended in s-DMEM, seeded onto the polycarbonate filters of the transwells at a density of 3×10^4 cells/cm^2 and further incubated under normal cell culture conditions. The growth media was replaced every other day and transepithelial electrical resistance (TEER) was monitored periodically by a Millicell® ERS meter (Millipore, Bedford, MA) connected to a pair of Ag/AgCl electrodes. The monolayer became confluent and ready for transport studies after about 5 days when the TEER reading reached a plateau.

2.3. Electron microscopy

Electron microscopy was used to examine the adherence of *L. lactis* to the C-33A monolayer. The C-33A cells were seeded onto a glass cover slip (13 mm in diameter) in a six-well plate at a concentration of 3×10^4 cells/ml and were allowed to grow to form monolayer. The monolayer was then washed twice with phosphate buffered saline (PBS), and added with 2 ml of the *L. lactis* in antibiotic-free and FBS-free s-DMEM (4×10^7 cfu/ml). The plate was incubated at normal cell culture conditions for 3 h. The monolayer was washed five times with PBS and then fixed with 2.5% glutaraldehyde in PBS for 1 h at room temperature. After being washed five times with PBS, the monolayer was fixed for 30 min with 2% OsO_4, washed five times with PBS