ELSEVIER

FEMS Microbiology Letters 247 (2005) 51–57

FEMS MICROBIOLOGY Letters

www.fems-microbiology.org

Flavoridin inhibits *Yersinia enterocolitica* uptake into fibronectin-adherent HeLa cells

Antonio Scibelli [a], Gianluca Matteoli [a], Sante Roperto [a], Elena Alimenti [b], Ludovico Dipineto [a], Luigi Michele Pavone [b], Rossella Della Morte [b,c], Lucia Francesca Menna [a], Alessandro Fioretti [a], Norma Staiano [b,c,*]

[a] *Dipartimento di Patologia e Sanità Animale*

[b] *Dipartimento di Strutture, Funzioni e Tecnologie Biologiche, Università di Napoli Federico II, Via F. Delpino 1, 80137 Napoli, Italy*

[c] *Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II, Napoli, Italy*

Received 15 February 2005; received in revised form 18 April 2005; accepted 18 April 2005

First published online 30 April 2005

Edited by S. Smith

Abstract

In this study, three structurally distinct disintegrins (flavoridin, echistatin, kistrin) were used as molecular probes to further characterize the molecular mechanisms underlying *Yersinia enterocolitica* infection of host cells. The activity of the three disintegrins on *Y. enterocolitica* uptake into fibronectin-adherent HeLa cells was evaluated at disintegrin doses which were non-cytotoxic and unable to induce cell detachment. Flavoridin resulted to be the most effective in inhibiting bacterial entry into host cells; echistatin was almost 50% less effective than flavoridin, whereas kistrin was definitely inactive. Our results suggest that $\alpha_5\beta_1$ integrin receptor, which binds flavoridin with higher affinity than the other two disintegrins, plays a major role in *Y. enterocolitica* uptake into HeLa cells. Furthermore, flavoridin binding to this integrin prevented the disruption of the functional complex FAK–Cas, which occurs in the *Y. enterocolitica* uptake process.

Keywords: Disintegrins; *Yersinia enterocolitica*; Uptake; HeLa cells; FAK; Cas; Paxillin

1. Introduction

The gram-negative bacteria *Yersinia* spp. infect both animals and humans, causing diseases ranging from mild gastroenteritis to bubonic plague. The bacteria resist the non-specific host defense and proliferate extracellularly in lymphatic tissues [1]. *Yersinia* spp. initiates disease through bacterial translocation across M cells into the Peyer's patches of the small intestine [2]. Efficient entry into both cultured mammalian cells and M cells requires the bacterial surface protein invasin [3]. Invasin binds at least five different β_1 integrin receptors ($\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$ and $\alpha_v\beta_1$) [1,2]. In the small intestine, only M cells display β_1 chain integrins to the intestinal lumen, thus explaining the specificity of the microorganism for this cell type [3].

Integrins fulfill multiple functions such as cell adhesion, signal transduction and cytoskeleton organization [4]. Focal adhesions are the sites where integrins link to intracellular cytoskeletal complexes and bundles of actin filaments [5]. Herein, the focal adhesion kinase FAK regulates the stability of focal adhesions which

* Corresponding author. Tel.: +39 081 2536108; fax: +39 081 2536097.

E-mail address: staianor@unina.it (N. Staiano).

0378-1097/$22.00
doi:10.1016/j.femsle.2005.04.024

have great significance for invasin-promoted uptake [6]. FAK binds integrin β_1 chain, and directly signals from the phagocytic cup to the cytoskeleton [7].

Upon intimate contact with the eukaryotic target cells, the bacteria secrete several proteins called Yops, by type III secretion machinery [8]. Once inside the host cells, Yops carry out disruption of signaling cascades which activate the processes of phagocytosis, cytokine release and respiratory burst [8]. Six Yop effectors have been identified [9]. YopH, in particular, is a protein tyrosine phosphatase which inhibits the actin cytoskeletal remodeling required for bacterial phagocytosis by dephosphorylating cell signaling proteins [9]. Substrates of YopH include focal adhesion-associated proteins such as FAK, Cas and paxillin, Src family kinases, Rac1, Arp 2/3 complex and Wasp family members [9,10].

Disintegrins are a family of low molecular weight proteins isolated from *Crotalidae* and *Vipiridae* snake venoms [11]. They typically contain an Arg-Gly-Asp (RGD) motif as their active site, except for barbourin containing a KGD sequence, and a new class of heterodimeric disintegrins such as EC3 and EMF10, containing MLD, VGD, and other recognition motifs [11]. RGD sequence is the cell attachment site of a large number of adhesive extracellular matrix (ECM), blood, and cell surface proteins [4]. Disintegrins bind with high affinity integrin receptors on cell surface, thus interfering with cellular functions such as signal transduction and cytoskeletal organization [12–14]. However, because of structural motifs other than RGD sequence present in their molecules, disintegrins show a high selectivity in their binding to different integrin receptors [15,16]. Additional determinants of disintegrin specificity for a given integrin may be the result of the RGD sequence-specific conformation or the aminoacid residues flanking the RGD site creating an extended RGD locus [16]. Thus, structurally distinct disintegrins differently affect cell adhesion and signaling [12–14].

In this study, three structurally distinct disintegrins (flavoridin, echistatin and kistrin) were used as molecular probes to further characterize *Yersinia enterocolitica* internalization process into host cells. Thus, disintegrin activity on *Y. enterocolitica* uptake into fibronectin-adherent HeLa cells was evaluated. Furthermore, the molecular mechanism by which disintegrins interfere with signaling pathways involved in the *Y. enterocolitica* infection of host cells was also investigated.

2. Materials and methods

2.1. Chemicals

Rabbit polyclonal anti-human FAK IgG (BC3), protein A– and protein G–agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); monoclonal mouse anti-paxillin IgG and monoclonal mouse anti-Cas antibody from Transduction Laboratories (Lexington, KY, USA); aprotinin, bovine serum albumin (BSA), Dulbecco's minimum Eagle's medium (DMEM), echistatin, flavoridin, horseradish peroxidase conjugated goat anti-(mouse or rabbit IgG) Ig, human plasma fibronectin, kistrin, leupeptin, orthovanadate, pepstatin, and RGDS (Arg-Gly-Asp-Ser) from Sigma (St. Louis, MO, USA); glutamine and trypsin from ICN Biomedicals (Aurora, OH, USA); fetal bovine serum (FBS) from Hyclone Laboratories (Logan, UT, USA).

2.2. Cell and bacterial cultures

HeLa cells were cultured in DMEM supplemented with 10% FBS, 1 nM sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 humidified incubator at 37 °C. Cells were harvested for propagation, cell attachment or uptake assay by treatment with 0.25% trypsin/0.02% ethylen-diamine-tetra acetic acid (EDTA) in phosphate-buffered saline (PBS), pH 7.2. Cells were washed with DMEM, and resuspended in complete DMEM for propagation, or in DMEM with 2% FBS for adhesion experiments, or in PBS for uptake assay. *Y. enterocolitica* cultures (9610 ATCC) were grown overnight at 26 °C in brain–heart infusion (Difco). Bacteria were washed once and resuspended in PBS before their use. Bacterial concentration was measured by their optical density at 600 nm [10].

2.3. Cell detachment assay

96-multiwell plastic dishes (Costar, Cambridge, MA, USA) were coated overnight at 4 °C by incubation with 100 μl human plasma fibronectin (10 μg/ml) diluted in PBS with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. After coating, dishes were treated with heat-denatured BSA 1% in PBS for 30 min at 37 °C to block free binding sites on the plastic. Freshly suspended cells (1×10^5) were plated onto fibronectin-coated wells, and allowed to adhere for 3 h at 37 °C. At the end of attachment period, non-adherent cells were removed by gentle washing with PBS, and different concentrations of echistatin, kistrin or flavoridin (0.1–50 μg/ml) in serum-free DMEM were added to the wells, and incubated for different time intervals (0–180 min). At the indicated time, detached cells were removed by washing with PBS; adherent cells were fixed, stained and counted [13]. The percentage of cell detachment induced by the disintegrin was calculated from the number of adherent cells in treated plates and those attached in control plates (untreated cells).