A synthetic polypeptide based on human E-cadherin inhibits invasion of human intestinal and liver cell lines by *Listeria monocytogenes*

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Internalin A is a surface protein of the facultative intracellular pathogen *Listeria monocytogenes* that interacts with the human host cell protein E-cadherin to facilitate invasion of epithelial cells. A single amino acid substitution at position 16 in mouse E-cadherin prevents this interaction. Synthetic polypeptides of 30 aa encompassing position 16 of human and mouse E-cadherin were tested for their ability to inhibit *in vitro* invasion of Caco-2, HepG2 and TIB73 cell lines by *L. monocytogenes*. Only the human-derived peptide was capable of inhibiting invasion in the human-origin Caco-2 and HepG2 cell lines. These findings demonstrate that small polypeptides can inhibit invasion of biologically relevant cell types by *L. monocytogenes in vitro* and may be potentially useful as therapeutic agents *in vivo*.

INTRODUCTION

As a facultative intracellular pathogen, *Listeria monocytogenes* is dependent upon the ability to invade and survive within host cells. In the case of human epithelial cells, invasion is mediated by interaction of the *L. monocytogenes* virulence protein internalin A (InlA) with E-cadherin, a transmembrane cell adhesion glycoprotein expressed on the surface of epithelial cells and other cell types (Lebrun *et al.*, 1996; Mengaud *et al.*, 1996a). The role that InlA plays in invasion of liver and other tissues is less clear. InlB, a closely related *Listeria* protein also containing leucine-rich repeat domains, is encoded within the same operon as *inlA* and may play a role in invasion of non-epithelial cells (Dramsi *et al.*, 1995; Khelef *et al.*, 2006; Pizarro-Cerda & Cossart, 2006). Polymorphic variations and mutations in InlA significantly alter the virulence potential of *L. monocytogenes* isolates (Nightingale *et al.*, 2005). A series of E-cadherin chimeras was developed, which identified the first extracellular domain as the portion of the molecule specifically interacting with InlA (Lecuit *et al.*, 1999). Whilst E-cadherin is evolutionarily conserved, species-specific polymorphisms exist that greatly affect the interaction with InlA and infectivity. A specific amino acid substitution in mouse E-cadherin has been associated with reduced oral infectivity in the mouse model compared with the guinea pig (Lecuit *et al.*, 2001). This single amino acid substitution of glutamic acid for proline at residue 16 renders mouse and rat E-cadherin ineffective as a ligand for InlA (Lecuit *et al.*, 1999), as demonstrated using an *in vitro*

model of cell invasion in which several cell lines expressing the mouse version of E-cadherin were uniformly refractory to *L. monocytogenes* invasion. Interference with the interaction of InlA and human E-cadherin has been shown to inhibit invasion of human epithelial cell lines. Antibodies specific for the leucine-rich repeat region of E-cadherin inhibit invasion (Mengaud *et al.*, 1996b). Supernatants from cultures of the Kato III human gastric cancer cell line secrete a truncated 25 kDa fragment of the N-terminal domain of E-cadherin that strongly inhibits invasion of Caco-2 cells (da Silva Tatley *et al.*, 2003). These results led us to investigate whether a synthetic polypeptide based on the region of human E-cadherin critical for binding InlA could inhibit cellular invasion by *L. monocytogenes* strains.

METHODS

Synthetic polypeptides. Standard Fmoc chemistry was used to synthesize two 30-mer peptides based on aa 1–30 of human and mouse E-cadherin sequences (Mohan *et al.*, 1995). These peptides differed only at position 16. The human peptide sequence was: H-DWVIPPISCPENEKGPFKPNLVQIKSNRDK-OH. In the mouse peptide, the proline at residue 16 was replaced with glutamic acid: H-DWVIPPISCPENEKGEPFKPNLVQIKSNRDK-OH.

Production of *L. monocytogenes* expressing green fluorescent protein (GFP). In order to assess the effects of these peptides on eukaryotic cell invasion, GFP-labelled *L. monocytogenes* strains were produced by transformation of *L. monocytogenes* strains Scott A and 12443 with the GFP plasmid pNF8 (Fortinea *et al.*, 2000). Scott A is a frequently used human clinical isolate (serotype 4b), whilst 12443 (serotype 1/2a) is a primate isolate that has been used to induce stillbirths in rhesus macaques (Smith *et al.*, 2003). For transformation of *L. monocytogenes*, 1–2 µg pNF8 plasmid DNA was electroporated

Abbreviation: GFP, green fluorescent protein.

Fig. 1. Effect of human and mouse E-cadherin synthetic polypeptides (10 and 100 $\mu\text{g ml}^{-1}$) on invasion of Caco-2 cells by GFP-labelled *L. monocytogenes* strain Scott A (m.o.i. = 13.2) as measured by flow cytometry and colony counts of internalized bacteria. The percentage of infected Caco-2 cells is indicated by the horizontal bar and number. The mean c.f.u. values for four replicate wells are also shown. Results are shown for baseline invasion (time 0) (a) and at 24 h (b). Values with the same superscript letter are not significantly different ($P < 0.05$).

into Scott A and 12443 cells using a Gene Pulser II (Bio-Rad) with previously described conditions and settings (Alexander *et al.*, 1990). The transformed cells were selected on brain heart infusion (BHI) agar containing 15 μg erythromycin ml^{-1} . After 24 h of growth, colonies were examined under long-wave UV light for GFP expression. Several of the most fluorescent colonies were selected, expanded in broth culture containing erythromycin and analysed by flow cytometry for GFP intensity. Isolates with the greatest fluorescence intensity were frozen in 50% glycerol and used for invasion experiments.

Cellular invasion assays. For invasion studies testing the effects of E-cadherin peptides, HPLC-purified peptides were dissolved in Hanks' balanced salt solution (HBSS; Invitrogen). GFP-labelled *L. monocytogenes* strains were grown for 18 h in BHI broth in the

presence of erythromycin, washed three times in HBSS and standardized at an OD_{550} of 0.5. Bacteria were then pelleted by centrifugation and resuspended in the same volume of RPMI 1640 (Invitrogen) without antibiotics and supplemented with 10% fetal bovine serum (FBS) in the presence of the indicated final concentration of peptide for 1 h prior to addition to eukaryotic cells. Caco-2 human epithelial cells, HepG2 human hepatocytes and TIB73 mouse hepatocytes (ATCC) were seeded into 24-well cell culture plates (Costar) at a concentration of 2×10^5 cells per well, 24 h prior to the addition of bacteria. Seeding the wells under these conditions routinely resulted in confluent monolayers after 24 h of growth. Cell wells were washed three times with antibiotic-free RPMI 1640 containing FBS and the bacteria/peptide suspension was added. Invasion was allowed to proceed for 1 h at 37 °C in 5% CO_2 . After

invasion, the cells were washed three times with antibiotic-free medium, followed by the addition of RPMI 1640 containing FBS and 10 µg gentamicin (Invitrogen) ml⁻¹. After 1 h of exposure to gentamicin, the level of cell invasion was determined by washing the cells with HBSS, followed by lysis with 1 ml 0.1 % Triton X-100 (Sigma) and plating of the cell lysate on tryptic soy agar (TSA). This time point was referred to as the baseline. Additional cells were assayed at 24 h to measure intracellular replication of bacteria. All invasion assays were performed in six replicate wells. To calculate invasion and replication, the c.f.u. count, based on the mean of duplicate TSA plates, from four replicate wells was averaged. Statistical comparisons were made between the mean c.f.u. of the four replicate wells using *t*-test analyses. Intact cells from the two additional replicate wells were harvested by treatment with trypsin/EDTA solution and used for determination of cell invasion by flow cytometric analysis of GFP-labelled *Listeria* cell invasion.

Flow cytometry. Caco-2 and HepG2 cells infected with GFP-labelled *Listeria* were analysed for infection by flow cytometry using a FACSVantage SE flow cytometer (Becton Dickinson) as described previously (Raybourne & Bunning 1994; Xie *et al.*, 2003). Briefly, the viable cell population was gated based on forward versus 90° light scatter parameters and the green fluorescence intensity of the gated population was used to identify *L. monocytogenes*-infected cells.

Statistical analysis. The mean c.f.u. per well was compared among four replicate wells of each treatment by analysis of variance. Tukey's studentized range test was used to compare mouse and human E-cadherin peptides and Dunnett's *t*-test was used to compare both E-cadherin peptides with controls (no peptide).

RESULTS AND DISCUSSION

Inhibition of invasion in Caco-2 cells

Experiments testing the ability of E-cadherin peptides to inhibit invasion of Caco-2 cells were repeated three times with similar results. Representative data are shown in Fig. 1. Flow cytometry of baseline samples indicated that exposure to the human E-cadherin peptide resulted in a large reduction in the percentage of Caco-2 cells that were positive for GFP-labelled *Listeria* compared with the results from Caco-2 cells exposed to the mouse E-cadherin peptide, which was similar to the control with no peptide (Fig. 1a). This effect was concentration-dependent, with 100 µg peptide ml⁻¹ showing a 99 % inhibition of

invasion, whereas 10 µg ml⁻¹ resulted in approximately 50 % inhibition. Results of colony counts (c.f.u.) paralleled this finding but were somewhat less sensitive in demonstrating the inhibitory effect of the lower concentration of human E-cadherin peptide. An approximate 10-fold reduction in c.f.u. was seen at the higher concentration, and there was a distinct dose effect on invasion, as measured by c.f.u., using the lower concentration of peptide. Statistically, both concentrations of human peptide were significantly different from the control (*P* < 0.05), whereas neither concentration of the mouse peptide was different from the control. The mouse and human peptides were significantly different from each other at both concentrations. Increasing the concentration of human E-cadherin peptide to 500 µg ml⁻¹ did not result in further inhibition of invasion (data not shown). At 24 h, there was an approximate 100-fold increase in the number of *Listeria* c.f.u. However, the relative difference between the human E-cadherin peptide and the other groups was maintained. Flow cytometry results at 24 h demonstrated that over 90 % of Caco-2 cells in all groups except the highest concentration of human E-cadherin peptide had become infected as a result of cell-to-cell spread of *L. monocytogenes* between confluent cells (Portnoy *et al.*, 2002) (Fig. 1b). Whilst there was also a greater than 10-fold increase in the percentage of infected cells after 24 h following treatment with the higher concentration of human E-cadherin peptide, the effects of inhibition of invasion by the peptide were still evident. These findings were replicated and extended to a primate isolate of *L. monocytogenes*, strain 12443, which is a different serotype (1/2a) (Table 1). These results also showed that, whilst there was replication (fold increase) of *L. monocytogenes* at 24 h in all groups, there was reduced replication in the human-derived peptide groups indicating a residual effect at 24 h. Statistical analysis of the invasion data at baseline again demonstrated a significant difference between the human and mouse peptides. As expected, statistical analysis indicated a significant difference between the control and human peptide-treated wells for both strains of *L. monocytogenes*. A small but statistically significant difference was also seen between the

Table 1. Effect of mouse and human sequence-based E-cadherin synthetic peptides on invasion of Caco-2 enterocytes by two *L. monocytogenes* serotypes

Values with the same superscript letter are not significantly different (*P* < 0.05).

E-cadherin peptide	Strain*	c.f.u. at baseline	Invasion (%)	c.f.u. at 24 h	Fold increase
0 (control)	Scott A	2.9 × 10 ⁵ (± 0.22 × 10 ⁵) ^c	11.2	2.7 × 10 ⁷ (± 0.44 × 10 ⁷) ^a	93
	12443	2.2 × 10 ⁵ (± 0.61 × 10 ⁵) ^a	13.2	3.1 × 10 ⁷ (± 0.66 × 10 ⁷) ^a	124
Mouse (100 µg ml ⁻¹)	Scott A	2.4 × 10 ⁵ (± 0.25 × 10 ⁵) ^a	9.2	2.3 × 10 ⁷ (± 0.35 × 10 ⁷) ^a	96
	12443	2.0 × 10 ⁵ (± 0.47 × 10 ⁵) ^a	10.5	2.6 × 10 ⁷ (± 0.48 × 10 ⁷) ^a	130
Human (100 µg ml ⁻¹)	Scott A	1.4 × 10 ⁴ (± 0.11 × 10 ⁴) ^b	0.5	9.5 × 10 ⁵ (± 1.50 × 10 ⁵) ^b	68
	12443	1.7 × 10 ⁴ (± 0.20 × 10 ⁴) ^b	0.9	1.4 × 10 ⁶ (± 0.06 × 10 ⁶) ^b	82

*Scott A was used at an m.o.i. of 12.4 and 12443 at an m.o.i. of 9.1.

Table 2. Effect of mouse and human sequence-based E-cadherin synthetic peptides on invasion of mouse hepatocytes by *L. monocytogenes* strain Scott A

Values with the same superscript letters are not significantly different ($P < 0.05$). Scott A was used at an m.o.i. of 9.6.

E-cadherin peptide	c.f.u. at baseline	Invasion (%)	c.f.u. at 24 h	Fold increase
0 (control)	$5.7 \times 10^4 (\pm 0.51 \times 10^4)^b$	2.95	$5.1 \times 10^6 (\pm 0.49 \times 10^6)^c$	89.5
Mouse (100 $\mu\text{g ml}^{-1}$)	$4.1 \times 10^4 (\pm 0.45 \times 10^4)^a$	2.12	$3.8 \times 10^6 (\pm 0.29 \times 10^6)^a$	92.7
Human (100 $\mu\text{g ml}^{-1}$)	$3.8 \times 10^4 (\pm 0.22 \times 10^4)^a$	1.97	$3.1 \times 10^6 (\pm 0.44 \times 10^6)^b$	81.6

control and mouse peptide with strain Scott A, but not with strain 12443. It should be noted, however, that the magnitude of difference between the mouse peptide and control, whilst statistically different, was very small relative to the human peptide and may be of questionable biological significance in the outcome of the *in vitro* infection. This effect with mouse-derived peptide occurred in one of four experiments involving human cell lines and may reflect a non-specific effect of peptide treatment or a weak interaction between the mouse peptide and the bacteria. The more important trend was that mouse and human peptides were significantly different in all experiments with human cell lines.

Inhibition of invasion in hepatocytes

To address the cellular specificity of the peptide inhibition of invasion, similar experiments were carried out with TIB73 mouse hepatocytes and the human hepatocyte line HepG2. Neither mouse nor human E-cadherin peptide influenced invasion of the mouse cell line by *L. monocytogenes* to the degree seen with Caco-2 and the human-derived peptide (Table 2). Statistically small but significant differences were seen with both the mouse and human peptides when compared with the control with no peptide; however, there was no difference between the mouse- and human-derived peptides. With HepG2 human hepatocytes, there was a higher degree of invasion than was seen with the Caco-2 intestinal cell line at an equivalent m.o.i. and an approximate sixfold inhibition of invasion with the human E-cadherin peptide (Table 3). As expected, the efficiency of invasion was greatly increased in human hepatocytes compared with mouse. Flow cytometry confirmed this observation (data not shown). Statistically significant differences were seen between the human peptide and control and

between the mouse and human peptides. These results indicated that the invasion process may be more efficient in HepG2 cells than in the Caco-2 human intestinal cell line. This may be relevant to infection *in vivo*, as the liver is a site of replication in systemic listeriosis (Gregory *et al.*, 1992).

Expression of E-cadherin on Caco-2 and HepG2 cells

As peptide inhibition of invasion is assumed to be specific for the InlA–E-cadherin interaction, we investigated expression of E-cadherin on adherent HepG2 and Caco-2 cells, as trypsin treatment to detach adherent cells can affect E-cadherin. HepG2 and Caco-2 cells attached to four-well glass culture slides (BD Biosciences) were stained with a phycoerythrin-conjugated monoclonal antibody specific for human E-cadherin (1 $\mu\text{g ml}^{-1}$; R&D Systems) and examined by laser-scanning cytometry (CompuCyte). Controls for non-specific binding of the antibody were Fc receptor blocking with normal human IgG and a phycoerythrin-conjugated isotype control antibody. Both cell lines were equally positive for E-cadherin (Fig. 2), indicating that increased E-cadherin expression was not the basis of increased invasion efficiency in HepG2 cells. The increased efficiency of invasion in hepatocytes may be a reflection of synergism between InlA and InlB, which is involved in invasion of hepatocytes and a number of other cell types. The *inlB* gene is contained within the same operon as *inlA* and may be co-transcribed with *inlA* under the control of the *prfA* transcriptional regulator (Lingnau *et al.*, 1995). InlB mediates invasion by co-opting host cell signalling pathways via interaction with the hepatocyte growth factor receptor Met, which is found on a large number of cell types (Shen *et al.*, 2000). A cell invasion study using *inlA* and *inlB* gene deletion mutants and the

Table 3. Effect of mouse and human sequence-based E-cadherin synthetic peptides on invasion of human hepatocytes by *L. monocytogenes* strain Scott A

Values with the same superscript letters are not significantly different ($P < 0.05$). Scott A was used at an m.o.i. of 13.7.

E-cadherin peptide	c.f.u. at baseline	Invasion (%)	c.f.u. at 24 h	Fold increase
0 (control)	$1.1 \times 10^6 (\pm 0.01 \times 10^6)^a$	42.3	$1.16 \times 10^8 (\pm 0.06 \times 10^8)^c$	105.5
Mouse (100 $\mu\text{g ml}^{-1}$)	$9.9 \times 10^5 (\pm 0.62 \times 10^5)^a$	38.1	$9.7 \times 10^7 (\pm 0.67 \times 10^7)^a$	98.0
Human (100 $\mu\text{g ml}^{-1}$)	$1.82 \times 10^5 (\pm 0.08 \times 10^5)^b$	7.0	$1.69 \times 10^7 (\pm 0.10 \times 10^7)^b$	92.9

Fig. 2. Immunofluorescent staining of Caco-2 and HepG2 cells with phycoerythrin-labelled anti-human E-cadherin monoclonal antibody (line histograms). The x-axis represents fluorescence intensity (log scale). The solid histograms are isotype control staining with phycoerythrin-labelled mouse IgG2.

same cell lines used in this study demonstrated that InlB alone mediated invasion of mouse hepatocytes, and that both InlA and InlB were required for human hepatocyte invasion (Dramsi *et al.*, 1995). Our results support this observation, as specific interference with InlA by the human E-cadherin peptide strongly inhibited human hepatocyte invasion, but the invasion of mouse hepatocytes was not affected to a similar extent.

Our results indicate that a relatively small peptide of approximately 3.5 kDa can effectively inhibit invasion of human enterocyte and hepatocyte cell lines. In addition to invasion inhibition by Kato III cell culture supernatants, it has been demonstrated that a recombinant human E-cadherin 25 kDa peptide inhibited invasion of Caco-2 cells by *L. monocytogenes* and that a mutant version of this peptide with a proline to glutamate substitution at position 16 was ineffective at inhibiting invasion (da Silva Tatley *et al.*, 2003). Our results support and extend these findings by demonstrating that a synthetic polypeptide of about one-seventh of this molecular mass can inhibit Caco-2 cell invasion. In addition, flow cytometric analysis using GFP-labelled *L. monocytogenes* provided independent direct evidence for inhibition of invasion. We also followed the infection for 24 h and demonstrated the residual effects of the human-derived peptide. The degree of inhibition achieved with the synthetic peptide appeared to be equivalent to or greater than that seen with the Kato III supernatants or the 25 kDa recombinant truncated human E-cadherin protein (da Silva Tatley *et al.*, 2003), indicating that the smaller synthetic peptide is capable of interfering efficiently with the receptor–ligand interaction between InlA and E-cadherin on host cells. It further suggests that the interaction with InlA may not involve tertiary conformational structure on the part of E-cadherin. It does not exclude a potential role for formation of multimers of the peptide as a result of interpeptide disulfide bonds, as there is a single cysteine in both the human and mouse peptides. As synthetic polypeptides can easily be produced and purified in relatively large amounts, they may provide a useful tool to study the mechanisms involved in InlA-mediated host cell invasion. In addition, the ability of the peptide to inhibit invasion in cell types

such as hepatocytes that involve InlB and InlA raises the possibility that it could be a useful novel therapeutic agent to modify systemic infection.

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