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PRO-APOPTOTIC PEPTIDES AS POTENTIAL THERAPY FOR PERITONEAL ENDOMETRIOSIS

Endometriosis is a common gynaecological disease associated with pelvic pain and infertility. Current treatments include oral contraceptives combined with non-steroidal anti-inflammatory drugs or surgery to remove lesions, all of which provide a temporary but not complete cure. Here we identify an endometriosis-targeting peptide that is internalized by cells, designated z13, using phage display. As most endometriosis occurs on organ surfaces facing the peritoneum, we subtracted a phage display library with female mouse peritoneum tissue and selected phage clones by binding to human endometrial epithelial cells. Proteomics analysis revealed the z13 receptor as the cyclic nucleotide-gated channel β3, a sorting pathway protein. We then linked z13 with an apoptosis-inducing peptide and with an endosome-escaping peptide. When these peptides were co-administered into the peritoneum of baboons with endometriosis, cells in lesions selectively underwent apoptosis with no effect on neighbouring organs. Thus, this study presents a strategy that could be useful to treat peritoneal endometriosis in humans.Endometriosis occurs only in higher primates, including humans and baboons. Spontaneous endometriosis reportedly occurs in about 25 % of captive baboons, and its prevalence increases with captivity duration [5]. Experiments in non-human primates show a clear positive correlation between endometriosis and a diet containing the chemical dioxin [6, 7], which may promote endometriosis by acting as an estrogen-like factor. In patients with endometriosis, many of the pathologic processes including inflammation, the immune response, angiogenesis and apoptosis are all favoured for promoting endometriosis in a manner dependent on steroid hormones – [8, 9]. Thus, in the past, most treatment was via therapeutics targeting steroid hormones and their receptors – [10, 11]. These drugs can only be administered for a short term due to side effects. Current first-line therapy is oral contraceptive pills, which halt an ovulation and suppress endometriosis tissue growth with minimum side effect – [12]. Oral contraceptive pills are also administered with nonsteroidal anti-inflammatory drugs, further reducing endometriosis-associated pain [13, 14]. Nonetheless, these treatments do not remove endometriosis, and patients with severe symptoms must undergo surgery. However, even then symptoms can re-

cur, requiring multiple surgeries in many cases [15]. Thus, it is critical to develop new strategies to cure this disease, particularly to prevent recurrence after surgery.

One proposed cause for endometriosis is through retrograde menstrual reflux of the endometrium that becomes implanted in regions of the pelvis [16], most commonly on the ovaries and areas facing the peritoneum [17, 18]. Thus, we hypothesized that a molecule specifically expressed on the endometrial surface could be targeted by a peritoneally injected drug if that marker is not expressed by other peritoneal surfaces. We report here the identification of such a reagent, namely, a 9-mer peptide that specifically binds to glandular epithelial cells of endometriosis in peritoneum. We also identified its receptor as the cyclic nucleotide-gated channel β39(CNGB3) and confirmed its expression in endometriosis. On the basis of these findings, we developed potential therapeutics for endometriosis targeting disease lesions at peritoneal surfaces and tested their activity in baboon endometriosis models. This study presents a strategy that could be useful to treat peritoneal endometriosis in humans.

Identification of peptides targeting peritoneal endometriosis.

As yet, there is neither an *in vitro* model nor an *in vivo* animal model available to study endometriosis, other than non-human primates. To identify a peptide that specifically binds to epithelial cells in endometriosis, we hypothesized that some, if not all, human endometrial adenocarcinoma cell lines would express cell surface proteins expressed in glandular epithelial cells in endometriosis. To devise a probe that specifically binds to the endometriosis surface but not to the surface of other organs facing the peritoneum, we undertook subtractive phage library screening [18, 19]. A T7 phage-based library (10^9clones, 10^{11}plaque-forming unit) of linear 9-mer peptides was injected into the peritoneal cavity of a female mouse to allow absorption of phage clones to the peritoneal surface *in vivo* for 1 h. The precleared library was recovered from peritoneal fluid and added to a monolayer of human endometrial adenocarcinoma Ishikawa cells cultured *in vitro*. We choose Ishikawa cells as this cell line shares characteristics with mature endometrial epithelial cells [20, 21]. We also wanted to identify a peptide internalized by endometrial glandular epithelial cells so that a drug conjugated with that peptide would penetrate target cells. We therefore incubated phage with live Ishikawa cells at 37 °C for 30 min to facilitate phage internalization. Selected phage clones were recovered after solubilizing cells with detergent and amplified in bacteria. After three rounds of subtractive library screening, the number of phage clones with Ishikawa cell-binding activity relative to the total number of added phage increased 10,000-fold.

The third screen-positive phage pool was overlayed on frozen tissue sections of endometriosis lesions surgically isolated from endometriosis patients. Immunohistochemistry using an anti-phage antibody showed positive signals on glandular epithe-

lial cells, particularly at apical cell surfaces in endometriosis. These results suggest that, despite differences between endometriosis and endometrial adenocarcinoma, the positive phage pool bound to the Ishikawa cancer cell line contained a clone or clones binding to endometrial glandular epithelial cells. Sequencing of insert DNAs from isolated phage clones revealed the deduced consensus sequence VRRAXNXPG (where X represents a varying amino-acid residue). The presence of the consensus sequence attests to the high specificity of selected clones. *In vitro* binding assays indicated that each clone bound to Ishikawa cells at higher efficiency than to control skin epidermoid carcinoma A431 cells. Among the clones, z13, which displayed the sequence VRRADNRPG, was the strongest binder. Z13 phage bound to endometrial adenocarcinoma SNG-II, RL95-2 and Hec1A cells but not to 431, prostate cancer PC3 or cervical cancer HeLa cell.

To confirm z13 peptide-binding activity, we chemically synthesized z13 peptide with an amino-terminal fluorescein isothiocyanate (FITC) tag and added to Ishikawa cells and control A431 cells . Fluorescence micrographs showed binding of FITC-z13 to Ishikawa cells, but not to A431 cells. Micrograph of Ishikawa cells showed a punctate cytoplasmic staining pattern, suggesting that z13 is internalized to endosomes.

Fluorescence micrographs of Ishikawa cells (left) and control A431 cells (right) overlayed with a synthetic z13 peptide tagged with fluorescein isothiocyanate (FITC) and left at 37 °C for 15 min. Scale bar, 50 μm. Visualization and isolation of the z13 peptide receptor. Left: cell surface proteins expressed on Ishikawa cells were biotinylated. Cell lysates were bound to z13 peptide-conjugated agarose beads, and bound proteins were eluted with irrelevant peptide (lane 1) or z13 peptide (lane 2). Biotinylated proteins in each eluate were detected by peroxidase-conjugated avidin and a luminescent peroxidase substrate. Right: silver staining of peptide-affinity-purified z13 receptor from endometriosis. Endometriosis tissues isolated from patients were homogenized, and microsome membrane fraction was prepared. Proteins solubilized with detergent were applied to a z13 peptide-conjugated agarose column, and bound proteins were eluted with irrelevant peptide (lane 1) or z13 peptide (lane 2). Proteins in each eluate in SDS-PAGE were detected by silver staining. (c) Fluorescence micrographs of HeLa cells transfected with control empty vector (upper row) or with an expression vector encoding CNGB3-MYC (lower row). Binding of FITC-z13 peptide (green) to HeLa cells transfected with mammalian expression vectors (a,d), immunostained with anti-MYC followed by Alexa 549 (red)-conjugated anti-mouse IgG antibody (b,e) and merged images including 4',6-diamidino-2-phenylindole (blue) to indicate nuclear staining (c,f). Scale bar, 20 μm.

Identification of the z13 peptide receptor.

To develop a clinically relevant therapeutic strategy, we searched for the z13 peptide receptor. To do so, Ishikawa cells were surface biotinylated and lysed, and then lysates were incubated with z13 peptide-conjugated agarose beads. Bead-bound materials were then eluted by z13 peptide, and biotinylated proteins detected by avidin blot, revealing a single 68-kDa protein. To identify this protein, the microsomal membrane fraction was prepared from endometriosis tissue surgically removed from patients. Membrane proteins solubilized with detergent were applied to a z13 peptide-affinity column and column-bound materials were eluted by z13 peptide. A silver-stained gel revealed a 68-kDa protein, and proteomic analysis identified the peptide sequence, QRTALYK, which is unique to the CNGB3 CNGB3 protein has six transmembrane domain [22]. A large part of this protein is buried in the lipid bilayer, the N- and C-terminal domains are cytoplasmic and presumably the z13-binding regions are extracellular. To assess binding of FITC-z13 to CNGB3, we transfected HeLa cells with a mammalian expression vector encoding a CNGB3–MYC fusion protein or with control empty vector. FITC-z13 did not bind to vector-transfected control cells. HeLa cells transfected with the CNGB3–MYC expression construct and stained by anti-MYC antibody showed an endosome-like pattern . When FITC-z13 was added to culture medium of CNGB3–MYC-expressing HeLa cells, FITC-z13 bound to sites marked by MYC epitope expression. The kinetics of FITC-z13 binding to CNGB3–MYC-expressing HEK293T cells showed a K_d of 9.759×10^{-6} M and a B_{max} of 0,9086 moles per receptor. These results indicate that recombinant CNGB3 expressed in a mammalian cell has z13 peptide-binding activity on the cell surface and internalized to endosome, supporting the hypothesis that CNGB3 is the z13 receptor.To examine CNGB3 protein expression in endometriosis, we generated a mouse monoclonal antibody against a peptide sequence of human CNGB3, corresponding to K [12, 14, 15] to P [1, 3] within the cytoplasmic domain. Antibody specificity was validated by immunostaining of CNGB3–MYC expressed in HeLa cells. This antibody robustly stained glandular epithelia of endometriosis in tissue sections. When we evaluated endometriosis tissue sections from 35 endometriosis patients, we found that 31 specimens showed strongly positive immunostaining and 4 showed weak/negative immunostaining. Peritoneal surfaces from cycling women without endometriosis were not stained by this antibody, whereas those from endometriosis patients were stained by this antibody, suggesting that endometrial cells are spread across a wide area on the peritoneum of endometriosis patients. Eutopic endometrial tissues at secretory and proliferative phases were weakly stained by this antibody. Immunohistochemistry of human tissues showed that this antibody did not stain the surface of organs facing the peritoneal cavity (data not shown).

Histology of hematoxylin and eosin-stained tissue sections revealed evidence of endometriosis in all three animals treated with a mixture of dKLAK-z13 and HLAH-z13 peptides. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays of endometriosis lesions collected from three untreated control baboons revealed no TUNEL positivity in gland tissue. By contrast, we found TUNEL-positive glands in tissues collected from all three baboons treated with the dKLAK-z13 and HLAH-z13 peptide mixture. Those signals were seen in glandular epithelial cells in ovarian endometriosis and in the lumen of endometrial gland in the omentum. No evidence of apoptosis was detected in cells facing peritoneum in liver, kidney, spleen, colon and stomach (data not shown). These results show overall, as proof of concept, that z13-targeted induced apoptosis occurred in endometriosis model in baboons *in vivo*.

A general strategy of translational research is to identify a gene product associated with a disease of interest and then develop drug targeting that molecule. However, in identifying peptides that target endometriosis but not non-endometrial cells facing the peritoneal cavity, we took a novel approach that does not require knowledge of a target. We also demonstrated that CNGB3 is the receptor for z13 peptide based on its expression pattern, not its function. We feel that analysing CNGB3 function in detail is not a priority for clinical application of this work.

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