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Uptake of *Marasmius oreades* agglutinin disrupts integrin-dependent cell adhesion

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ABSTRACT

Background: Fruiting body lectins have been proposed to act as effector proteins in the defense of fungi against parasites and predators. The *Marasmius oreades* agglutinin (MOA) is a lectin from the fairy ring mushroom with specificity for Galα1-3Gal containing carbohydrates. This lectin is composed of an N-terminal carbohydrate-binding domain and a C-terminal dimerization domain. The dimerization domain of MOA shows in addition calcium-dependent cysteine protease activity, similar to the calpain family.
Methods: Cell detachment assay, cell viability assay, immunofluorescence, live cell imaging and Western blot using MDCKII cell line.
Results: In this study, we demonstrate in MDCKII cells that after internalization, MOA protease activity induces profound physiological cellular responses, like cytoskeleton rearrangement, cell detachment and cell death. These changes are preceded by a decrease in FAK phosphorylation and an internalization and degradation of β1-integrin, consistent with a disruption of integrin-dependent cell adhesion signaling. Once internalized, MOA accumulates in late endosomal compartments.
Conclusion: Our results suggest a possible toxic mechanism of MOA, which consists of disturbing the cell adhesion and the cell viability.
General significance: After being ingested by a predator, MOA might exert a protective role by diminishing host cell integrity.

1. Introduction

Mushrooms are known to contain a wide variety of lectins with different structures and specificities [1]. Cytoplasmic fungal lectins, also referred to as fruiting body lectins, are part of the fungal defense system against parasites and predators [2]. When feeding on the content of the fungal cell, predators such as fungivorous nematodes ingest the cytoplasmic lectin, which then induces toxicity by an unknown mechanism after binding to specific glycans in the intestine of the worm [2–5].

The *Marasmius oreades* agglutinin (MOA) is a lectin from the fairy ring mushroom that was first reported to agglutinate blood group B erythrocytes [6]. The lectin has a dimeric structure composed of 293-residue protomers and specifically recognizes Galα1,3-containing structures, exhibiting the highest affinity for the branched blood group B trisaccharide Galα1,3(Fuc-α1,2)Gal [7,8]. Crystal structures of MOA in complex with two carbohydrate ligands revealed an N-terminal ricin B-chain like domain with three carbohydrate-binding sites and a C-terminal domain that is involved in dimerization [9,10]. The latter domain shows structural homology to enzymatically active proteins, and has been reported to be a calcium-dependent cysteine protease domain similar to the calpain and papain families [11,12].

MOA has been shown to exhibit a cytotoxic activity towards nematodes, which is dependent on the binding to plasma membrane glycosphingolipids and a cysteine protease activity [11]. Recently,

Abbreviations: MOA, *Marasmius oreades* agglutinin; MDCKII, Madin–Darby canine kidney strain II; FAK, Focal adhesion kinase; BAX, BCL-2-associated X protein; HUS, Hemolytic uremic syndrome; CME, Clathrin-mediated endocytosis; GSLs, Glycosphingolipids; DMEM, Dulbecco's Modified Eagle Medium; FCS, Fetal calf serum; PMP, D-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol; StxB, Shiga toxin B-subunit; wt, Wild-type; FACS, Fluorescence-activated cell sorting; PBS, Phosphate buffered saline; PNPG, 4-Nitrophenyl α-D-galactopyranoside; PFA, Paraformaldehyde; BSA, Bovine serum albumin; PEI, Polyethyleneimine; HBSS, Hawks buffer saline solution; RIPA, Radio-immunoprecipitation assay; ECM, Extracellular matrix; Tf, Transferrin; ER, Endoplasmic reticulum.

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Cordara *et al.* have demonstrated that MOA induces cytotoxicity in NIH/3 T3 cells, which correlates, at least in part, with its proteolytic activity [13]. Moreover, human BAX (BCL-2-associated X protein) has been identified as a potential target of MOA. In a separate study, MOA parenterally administered to mice caused symptoms comparable to the human Hemolytic Uremic Syndrome (HUS), going along with *in vitro* endothelial cell detachment [14]. However, no clear link has been established between cell detachment and the cysteine protease activity of MOA.

Cell adhesion is essential for tissue integrity. Processes that modify adhesion are tightly regulated by a wide range of proteins [15,16]. Abnormal disruption of adhesion attenuates nutrient and growth factor access and may induce cell death [15,17].

In this study, we investigated the possible role of the cysteine protease activity of MOA in the fungal defense system by studying its effect on cell adhesion in MDCKII cells. First, we demonstrate a cell detachment effect induced by MOA that is dependent on carbohydrate binding and cysteine protease activity, ultimately leading to cell death. Second, we show that cell detachment in response to MOA is preceded by disruption of the cytoskeleton, reduction of focal adhesion kinase (FAK) phosphorylation, internalization and degradation of β1-integrin. Finally, we show that MOA is mainly internalized by clathrin-mediated endocytosis (CME) and that it accumulates in late endosomal compartments. Our results suggest that MOA impairs cell survival by disturbing integrin-dependent cell adhesion signaling of the host in order to protect fungi against parasites and predators.

2. Materials and methods

2.1. Cell culture, generation of stable cell lines and depletion of glycosphingolipids (GSLs)

Madin–Darby canine kidney strain II cells (MDCKII, [18]) were grown at 5% CO_2 in DMEM (Dulbecco's Modified Eagle Medium, Life Technologies) containing 4.5 g/l glucose supplemented with 10% fetal calf serum (FCS) and 4 mM L-Glutamine. For the generation of a Gb3-expressing stable cell line, MDCKII cells were transfected with a plasmid encoding Gb3-synthase and a Geneticin resistance. After selection with 1000 μg/ml Geneticin, positive clones were maintained in DMEM containing 4.5 g/l glucose supplemented with 500 μg/ml Geneticin, 10% FCS and 4 mM L-Glutamine. For GSL depletion, cells were passaged for three days in the presence of 5 μM of the glucosylceramide synthase inhibitor D-threo-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP, Santa Cruz Biotech) [19,20]. To control the depletion of GSLs, we used Cy5-labeled B-subunit of Shiga toxin (StxB-Cy5), which is known to interact specifically with the glycosphingolipid Gb3. StxB was purified as previously described [21].

2.2. Protein expression, purification and labeling

Wild-type (wt) MOA and the variant MOA(C215A) were expressed recombinantly in *Escherichia coli* and purified as previously described [7,8]. For fluorescence microscopy analysis and fluorescence activated cell sorting (FACS) measurements, both molecules were conjugated to activated Cy2, Cy3 or Cy5 dyes with kits (Amersham Life Sciences) according to the manual.

2.3. Cellular assays

For the cell detachment assay, cells were seeded (40,000 cells/well in a 24-well plate) and allowed to adhere overnight. The cells were washed with phosphate buffered saline (PBS) and fresh medium was added together with MOA. For the cysteine protease inhibition with E-64 (Sigma Aldrich) and for the blocking of MOA binding sites with soluble carbohydrates (4-nitrophenyl α-D-galactopyranoside, PNPG, Sigma Aldrich), MOA and E-64, or MOA and PNPG, were pre-incubated together for 10 min at room temperature before being added to the cells. As positive control, cells were washed with PBS, and 0.05% of trypsin-EDTA (Life Technologies) was added for 15 min at 37 °C to the cells. The number of detached cells per well was quantified by flow cytometry (Gallios, Beckman Coulter). For the cell viability assay, live cells were quantified by trypan blue exclusion (Sigma Aldrich).

2.4. Caspase assay

The Caspase-Glo® 3/7 assay (Promega) was used to quantify MOA protease activity. The reagent was added to MOA-containing DMEM and luminescence was measured using a plate-reader (BioTek).

2.5. Cell binding and internalization assay

After 1 h of stimulation with Cy2-labeled MOA, cells were washed with PBS, detached from culture dishes and distributed equally to two tubes. For measuring extra- and intracellular MOA fluorescence, cells were measured immediately by flow cytometry using a FACS-Gallios (Beckman Coulter). For measuring only intracellular MOA fluorescence, cells were suspended in 0.4% Trypan Blue for 1 min to quench any residual cell surface fluorescence [22,23], then intracellular MOA-Cy2 fluorescence was measured immediately by flow cytometry.

2.6. Immunofluorescence

Immunofluorescence studies were carried out as previously described [24]. Briefly, cells grown overnight on 24-well cover glasses (Menzel, 12 mm) were treated with Cy2/Cy3-labeled MOA in FCS-containing medium and fixed in 4% paraformaldehyde (PFA) at 4 °C for 10 min. Cells were washed and incubated with NH_4Cl at room temperature for 15 min before being permeabilized with PBS containing 0.02% saponin and 0.2% bovine serum albumin (BSA). Cells were stained with either anti-EEA1 (1:50, BD Transduction Laboratories), anti-Calnexin (1:100, Enzo Life Science), anti-CTR 433 (1:100, kind gift of Michel Bornens, Curie Institut), anti-Giantin (1:100, Abcam), anti-TGN46 (1:100, Sigma Aldrich), anti-Transferrin receptor (TfR, 1:100, Life Technologies), anti-Rab11 (1:100, Cell Signaling), anti-Lamp1 (1:200, BD PharMingen), anti-Rab7 (1:100, Santa Cruz Biotech) or anti-β1 integrin (1:100, R&D Systems) antibodies diluted in permeabilization buffer, followed by either donkey anti-mouse Cy3-labeled secondary antibody (1:100, Jackson ImmunoResearch), donkey anti-rabbit Cy3-labeled secondary antibody (1:100, Jackson ImmunoResearch) or donkey anti-goat Cy3-labeled secondary antibody (1:100, Jackson ImmunoResearch) diluted in permeabilization buffer. Nuclei were stained using DAPI (300 nM, Life Technologies).

2.7. Transfection and confocal microscopy

Cells were imaged on a confocal microscope (Nikon Eclipse Ti-E with A1R confocal laser scanner, 60× oil objective, NA = 1.49). For live-cell-imaging, cells were transfected using polyethyleneimine (PEI, linear, MW: 25 kDa, Polyscience) as described elsewhere [25]. The medium was exchanged 5 h post-transfection and cells were imaged 30 h post-transfection. After being washed once with PBS with Ca^{2+}/Mg^{2+} at 37 °C, cells on glass coverslips were mounted on a coverslip holder with warm (37 °C) HBSS solution (Hawks Buffer saline solution, PAN Technology) supplemented with 10 mM HEPES, 4.5 g/l glucose, 1% FCS and 4 mM L-glutamine. Before starting measurements, the recording medium was exchanged with fresh recording medium containing MOA-Cy5 (10 μg/ml). Measurements were carried out at 37 °C for 25 min. Image acquisition and co-localization analysis were performed with NIS-Elements (Nikon).