Key Messages

1. We have generated monoclonal antibodies against the SARS coronavirus (SARS-CoV) X1/3a protein (3a), which are suitable for western blotting, immunocytochemistry, and immunohistochemistry.

2. We have established and characterised an in-vivo 3a transgenic Drosophila model, and demonstrated its usefulness in studying SARS-CoV 3a gene function.

3. We validated our in-vivo findings on 3a gene function in mammalian Vero E6 cells.

4. Our findings raise the possibility of using ion channel blockers as a novel approach to suppress SARS-CoV–induced cell death.

Introduction

SARS coronavirus (SARS-CoV) is the aetiological agent that caused a global outbreak of atypical pneumonia in 2003. To date, at least 14 open reading frames have been identified in the SARS-CoV genome; unravelling the molecular functions of these SARS-CoV gene products could provide a more complete understanding of SARS-CoV pathogenesis. The SARS-CoV 3a locus encodes a 274 a.a. protein. Expression of 3a is detected in patients’ intestinal surface enterocytes and pneumocytes. The 3a protein preferentially localises to the Golgi apparatus and cell surface in both transfected and virus-infected cells. The 3a protein is predicted to possess a short extracellular N-terminal head, three transmembrane regions, and a long intracellular C-terminal tail. Several conserved domains are found in 3a, including: a cysteine-rich domain, a Yxxφ domain, and a diacidic domain. The Yxxφ and diacidic domains are responsible for the intracellular protein trafficking of 3a, while the cysteine-rich domain is known to affect 3a self homo- and hetero-dimerisation, which is also crucial for its ion channel activity. It has been demonstrated that SARS-CoV induces apoptosis in infected cells, and a number of SARS-CoV proteins were reported to be pro-apoptotic. Furthermore, evidently apoptosis can initiate viral cytopathic effects in SARS-CoV–infected cells. In this project, we investigated the pro-apoptotic property of 3a, both in vivo and in vitro.

Methods

This study was conducted from January 2005 to December 2006.

BALB/c mice were immunised intraperitoneally with KLH-conjugated 3a peptide in complete Freund’s adjuvant (Sigma-Aldrich), and followed by monthly intraperitoneally boosts using the same antigen in incomplete Freund’s adjuvant (Sigma-Aldrich). Mice showing high serum antibody titers to the antigen were given a final booster injection. The mice were sacrificed 3 days later, and the spleens harvested. Fusion of the spleen cells with a nonproducer myeloma line was carried out.

Fly strains were grown at 29ºC on standard cornmeal medium supplemented with dry yeast. The UAS-3a transgene produces a full-length non-tagged 3a protein, whereas the UAS-EGFP-3a transgene produces a full-length 3a protein with EGFP fused to its N-terminus. The pUAST-3a-WT construct was then used as a template to generate three 3a mutant constructs: cysteine-rich (3a-CS; C127S, C130S, C133S), Yxxφ (3a-YA; Y160A), and diacidic (3a-DE; E171A, D173A).

African green monkey kidney cell line Vero E6 was maintained at 37ºC in Dulbecco’s modified Eagle’s medium (Invitrogen), containing 10% heat-inactivated foetal bovine serum (Gibco-BRL), streptomycin (100 g/mL), and penicillin (100 U/mL). Cells were seeded onto 24-well plates 24 hours prior to transient transfection.

Results

We performed detailed characterisation on three clones of monoclonal antibodies
(X61, X98 and X103) raised against the SARS-CoV 3a peptide antigen, mainly using clone X98 in this study. To investigate 3a function in vivo, the wildtype 3a gene (3a-WT) of the CUHK-W1 SARS-CoV isolate (AY278554) was used to generate 3a-WT and EGFP-3a-WT transgenic flies. When misexpressed in Drosophila, the 3a-WT protein displayed a punctate cytoplasmic subcellular localisation and caused external eye malformation in adult flies. As apoptosis has been reported in SARS-CoV–infected cells and the dysregulation of apoptotic cell death in Drosophila results in eye malformation, we investigated whether the 3a-induced eye malformation phenotype was related to apoptotic cell death. We detected increased numbers of apoptotic cells in 3a-WT–expressing flies as compared to controls. When 3a-WT was coexpressed with anti-apoptotic genes, the eye malformation phenotype was mostly suppressed and the number of apoptotic cells was considerably reduced. Our data clearly demonstrate that the SARS-CoV 3a-WT gene is pro-apoptotic.

We then performed site-directed mutagenesis on the cysteine-rich region (3a-CS; C127S C130S C133S), YXXψ (3a-YA; Y160A) and diacidic domains (3a-DE; E171A D173A), so as to investigate the significance of these regions on the pro-apoptotic function of 3a. Apoptotic cell staining was performed to assess the pro-apoptotic potential of the 3a mutants. In contrast to 3a-WT–expressing flies, relatively few apoptotic cells were detected in all three 3a mutants (unpublished). Further expression of each of these 3a mutants produced only a mild eye malformation phenotype compared to 3a-WT, consistent with 3a mutants being relatively less potent than their wildtype progenitor in inducing cell death. Our data clearly show that the cysteine-rich, YXXψ and diacidic domains are crucial for 3a’s pro-apoptotic function.

As 3a has been shown to form ion channels on membranes, we investigated the relationship between 3a’s channel activity and its pro-apoptotic function. We fed 3a-WT–expressing flies with barium chloride (barium [Ba] is a known ion channel blocker) and found that the numbers of apoptotic cells were considerably reduced when compared to the untreated control group (unpublished). Our findings therefore establish a link between the pro-apoptotic property and ion channel activity of 3a.

We then validated our data generated from the transgenic fly model in mammalian Vero E6 cells, and to further investigate the molecular mechanisms of 3a functions (with an emphasis on dissecting its relationship to ion channel activity and pro-apoptotic function). Similar to the fly data, we showed that Ba significantly suppressed cell death induced by 3a-WT in Vero E6 cells (unpublished). To determine the involvement of 3a’s channel activity in caspase-dependent apoptosis, we simultaneously treated 3a-WT–transfected Vero E6 cells with both Ba and caspase inhibitor; such drug co-treatment showed no further suppression of cell death (unpublished). These findings show that the ion channel activity of 3a-WT mainly regulates caspase-dependent apoptosis (unpublished).

Discussion

We generated and characterised several clones of 3a monoclonal antibodies; X98 and X61 are two that yielded high titers and specificity toward the 3a protein. Clone X98 was used primarily in our study; this clone was shown to be suitable for Western blotting, immnocytochemistry, and immunohistochemistry. We found that the 3a-WT protein displayed a punctate cytoplasmic localisation in Drosophila cells, comparable to the pattern observed in SARS-CoV–infected cells. We observed that the expression of 3a-WT protein caused external eye malformation. When EGFP was expressed in the fly eye as a control, no morphological eye changes could be detected. This suggests that at least to a certain extent, SARS-CoV protein can function in invertebrates as it does in mammalian cells. Together with its short life cycle and evolutionary gene pathway conservation (including the apoptotic cell death pathways), Drosophila therefore serves as a faithful complementary in-vivo experimental model to mammals, for the investigation of SARS-CoV in relation to viral gene functions.

By means of genetic interaction studies, we demonstrated that the eye malformation phenotype caused by 3a-WT protein expression was related to the caspase-dependent cell death pathway. Furthermore, the extent of apoptosis was ameliorated when anti-apoptotic genes such as caspase inhibitor were coexpressed with 3a. This prompted us to further investigate the protein domains in 3a that govern its pro-apoptotic property. By means of site-directed mutagenesis, we identified several regions essential for 3a-WT to execute its pro-apoptotic function (unpublished). We further focused our study on the cysteine-rich domain of 3a. It has been shown that 3a-WT protein possesses ion channel activity. We first demonstrated that when 3a-WT–expressing flies were fed the ion channel blocker Ba in vivo, apoptosis was largely suppressed (unpublished). Our data clearly show that 3a-WT–induced apoptosis is related to the disturbance of intracellular ion homeostasis. Accordingly, perturbation of intracellular ion flux in SARS-CoV–infected cells could therefore be one of its pathogenic mechanisms.

We used mammalian Vero E6 cells to validate our findings obtained from transgenic Drosophila, to further investigate the relationship between 3a’s ion channel activity and its pro-apoptotic property. After using multiple methods to confirm the pro-apoptotic role of 3a-WT in Vero E6 cells, we demonstrated involvement of caspase-8 and -9 activation in 3a-induced cell death (unpublished). In addition, we showed that the cysteine-rich, YXXψ and diacidic domains are all responsible for caspase-dependent apoptosis (unpublished). We further demonstrated that ion channel blockers can effectively inhibit caspase-dependent cell death in Vero E6 cells. This again demonstrates the consistency of data obtained from both invertebrate and
mammalian models. Interestingly, we showed that 3a-WT induces both caspase-dependent and -independent cell death in Vero E6 cells (unpublished). In the future it might be important to further delineate details of non-caspase-dependent apoptosis of 3a, both in vitro and in vivo.

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References