Anti-Apoptosis Engineering Using a Gene of *Bombyx mori*

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Abstract

We have previously shown that the addition of silkworm hemolymph to a culture medium increases the longevity of insect and mammalian cells by inhibiting apoptosis. This indicates that the component which inhibits apoptosis is contained in the silkworm hemolymph, The apoptosis-inhibiting component was isolated from silkworm hemolymph and characterized in our previous study. A database search using the N-terminal amino acid sequence of this component as a template resulted in a 95% homology with a low molecular weight lipoprotein, the so called '30K protein' of unknown function. In this study, the 30K protein gene was expressed in mammalian and insect cells to confirm the apoptosis-inhibiting effect. The overexpression of 30K protein in mammalian cell inhibited the staurosporin-induced apoptosis by the prevention of the activation of caspase 3. Using an *Autographa californica*nuclear polyhedrosis virus (AcNPV) system, the 30K protein was overexpressed also in insect cells. The expression of the 30K protein increased the longevity of baculovirus-infected insect cells by inhibiting apoptosis. These results suggest that the 30K protein is a novel anti-apoptotic protein.

Introduction

Apoptosis is physiological cell death, which morphologically distinguishable from necrosis. Necrosis is typified by cell swelling, mild clamping of chromatin, and lysis which results in the release of cell constituents whereas apoptosis is accompanied by condensation of nuclei and cytoplasm, loss of microvilli and convolution of plasma membranes, as well as nuclear and cell segmentation.[1] Programmed cell death occurs in most animal tissues to eliminate harmful cells or those produced in excess and it is mediated by apoptosis.[2] Insect hemolymph was used as a culture medium in the early stages of insect tissue culture. On the basis of the chemical analysis of insect hemolymph, a synthetic medium was formulated for insect cell culture. However these days, fetal bovine serum (FBS) has replaced insect hemolymph as a supplement in insect cell medium. We reported that the production of recombinant protein in an insect cell-baculovirus system was increased by supplementing the medium with silkworm hemolymph. We also found that the silkworm hemolymph increased host cell longevity by inhibiting baculovirus-induced insect cell apoptosis.[3,4] This indicates that the component, which inhibits the apoptosis is contained in the silkworm hemolymph. In

previously study,[5] the apoptosis-inhibiting component of silkworm hemolymph was isolated and characterized. A database search using the N-terminal amino acid sequence of this component as a template resulted in a 95% homology with a low molecular weight lipoprotein, the so-called '30K protein' of unknown function. In this study, we confirmed that the overexpression of 30K protein in mammalian cells and insect cells also inhibits apoptosis.

Materials and methods

Mammalian cell culture

Human embryonic kidney (HEK293) cells were cultivated in complete DMEM (Dubecco's modified Eagle's medium, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), HEPES, $NaHCO_3$ (2.02 g/L), and penicillin/streptomycin (Gibco). Chinese Hamster ovary (CHOK1) cells were cultivated in MEM (Minimum essential medium, Jeil Biotechservices Inc.) supplemented with 10% fetal bovine serum (FBS, Gibco), L-glutamine, non-essential amino acids and penicillin/streptomycin (Gibco). Both cells were maintained at 37°C in humidified air atmosphere with 5% CO_2.

Plasmid construction and transfection

30Kc6, one of cDNA clones of 30K protein was kindly provided by prof. Susumu Izumi (Dept. of Biology, Tokyo Metropolitan University). The 30K protein gene obtained as a 900bp EcoRI fragment from the pBm30Kc6RTP was cloned into the mammalian expression vector pcDNA3 (Invitrogen). pcDNA3/30K and pcDNA3 (vector alone) were transfected into mammalian cells by the LipofectAMINE Reagent (Gibco) according to the manufacturer's instructions. G418 (500 μg/mL, Gibco) was as a selection pressure for the establishment of stable cell lines expressing 30K protein. The expression of 30K protein was analyzed by RT-PCR from total cellular RNA of transfected cells.

Insect cell culture and baculovirus infection

Spodoptera frugiperda (*Sf* 9) cells were cultivated in Grace medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 0.35 g/L $NaHCO_3$, and antibiotic-antimicotic (Gibco) at 28°C. Cells were infected at MOI (Mutiplicity of infection) of 5 or 10 during the exponential growth phase with the recombinant baculovirus (*Autographa californica* nulear polyhedrosis virus, AcNPV).

Construction of recombinant baculovirus producing 30K protein

The obtained 732bp-BamHI and XhoI fragment of 30K protein gene by PCR from the pBm30Kc6RTP was cloned into the pBlueBacHis2 (pBBH) baculovirus transfer vector (invitrogen). The recombinant baculovirus producing 30K protein under control of polyhedrin promoter was also obtained by cotransfecting pBBH/30K with wild type AcNPV DNA (Bac-N-Blue baculovirus DNA; Invitrogen). Isolated pure recombinant virus stocks were amplified in *Sf* 9 cells and the titer of amplified virus was determined

by end-point dilution method. The expression of 30K protein was analyzed by RT-PCR from total cellular RNA of infected *Sf* 9 cells and immunoblotting.

Apoptosis induction and assay

Staurosporine was used as apoptosis-inducer for mammalian cells. It was added at final concentration of 300nM in the each mammalian cell culture media. Baculovirus-induced insect cell apoptosis was primarily assayed as host cell viability by trypan blue exclusion test. Flow cytometric analysis of each apoptotic cell was performed for quantification of apoptosis.

Results and discussion

30K protein expression in mammalian cell and its role on anti-apoptotic mechanism

HEK293 cells were transiently transfected with the 30K protein expression construct and followed by a staurosporine treatment. The 30K protein expression in HEK293 cell was analyzed by RT-PCR. Flow cytometric analysis of apoptosis of the transiently expressed cells treated with staurosporine for 48h after transfection was carried out. The fractions of apoptotic cells in the sub-G1 phase were 61.92%and 28.4% in the cells transfected with the vector control and the 30K protein expression construct, respectively. Also, the cell lines expressing 30K protein were established using the CHOK1 cell transfected with indicated plasmids. Stable transfectants were selected in the medium containing G418. The 30K protein expression in transfectants was analyzed by RT-PCR. Each of the vector-transfected clone and three 30K-transfected clones was treated with staurosporine at concentration of 300nM for 12h, and the apoptosis was assayed. The fraction of apoptotic cells in the 30K-transfected clones was significantly less than that of non-transfected control or vector-transfected clone (Fig. 1). This means that the 30K protein inhibits the apoptosis similar to the addition of silkworm hemolymph to a culture medium. Also, the expressed 30K protein of transfectants could prevent caspase 3 activation induced by staurosporine as results of fluorogenic caspase 3 assay on 30K-transfected clones, normal cell (non-transfected control) and vector-transfected clone (Fig. 2).

30K protein expression in insect cell by recombinant baculovirus

The PCR analysis was performed from the extracted DNA of the putative recombinant obtained by cotransfecting pBBH/30K with wild type AcNPV DNA using primers on the region of polyhedrin promoter to confirm the pure recombinant baculovirus (AcNPV/30K). The 30K protein expression was also confirmed by RT-PCR from the total cellular RNA of AcNPV/30K-infected *Sf* 9 cells and immunoblotting using anti-Xpress on expressing 30K protein containing anti-Xpress antibody epitope. *Sf* 9 cells were infected with the AcNPV/30K or recombinant baculovirus producing β-galactosidase under control of polyhedrin promoter (AcNPV/β-gal) and cell viability

was determined at various times postinfection. The viability of cells infected with AcNPV/β-gal decreased exponentially 3 days after the infection. In contrast, the viability of cells infected with AcNPV/30K showed the little decrease until 4 and 5 days after the infection (Fig. 3). This increased cell lonevity is caused by the inhibition of host cell apoptosis induced by baculovirus, and the extent of apoptosis could be quantitatively analyzed by the flow cytometric method. The percentage of a sub-G1 phase was 61.26% and 42.82% 5 days after infection in the cells infected with AcNPV/β-gal and AcNPV/30K, respectively (Fig. 4). This result also indicates that the expressed 30K protein in insect cell inhibits the apoptosis.

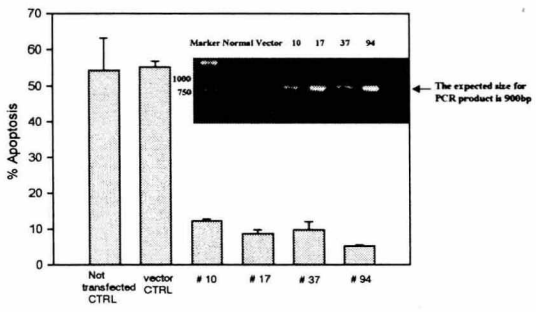

Figure 1. Effect of 30K protein expression on STS-induced CHOK1 cell apoptosis

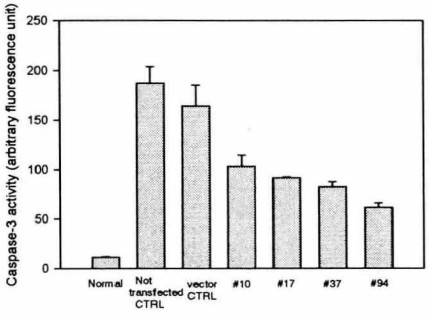

Figure 2. Effect of 30K protein expression on caspase 3 activity

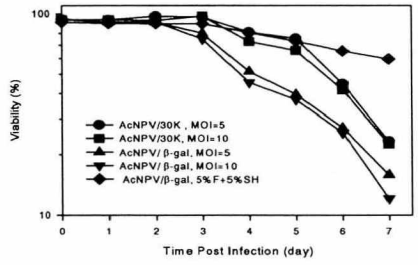

Figure 3. Comparison of host cell longevity infected with AcNPV/β-gal and AcNPV/30K

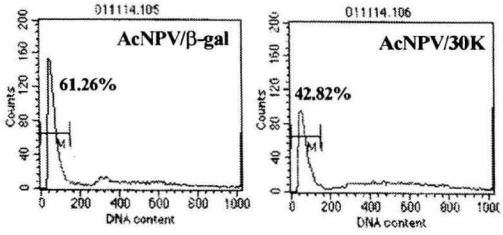

Figure 4. Flow cytometric analysis of *Sf* 9 cells infected with AcNPV/β-gal and AcNPV/30K (5 days after infection)

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