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Overexpression of Adenine Nucleotide Translocase Induces Apoptosis in *Drosophila* Cultured Cell

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The adenine nucleotide translocase (ANT) is a major component of mitochondrial permeability transition pore (MPTP) and catalyzes the exchange of adenine nucleotides across the mitochondrial inner membrane. It has recently been known that ANT is required for apoptosis mediated by Bax in mammal. However, in *Drosophila*, the study of ANT function is rarely known. The *Drosophila* ANT genes are duplicated tandemly, sesB and Ant2 respectively. In this study, using a GAL4-UAS system, we investigated the gain-of-function of sesB and Ant2 gene in *Drosophila* Kc cells. We confirmed overexpression of sesB and Ant2 gene in *Drosophila* Kc cell transiently transfected with the constructed plasmids pUAST-sesB or pUAST-Ant2, and pAc-GAL4 through RT-PCR. Subsequently, we observed apoptosis in *Drosophila* cultured cells transfected with the plasmid pAc-GAL4/pUAST-sesB or pAc-GAL4/pUAST-Ant2. The present results indicate that *Drosophila* ANT overexpression lead to apoptosis and suggest that dysregulation of ANT expression may be also associated with apoptosis.

Key Words: Adenine nucleotide translocase (ANT), Apoptosis, GAL4-UAS system, *Drosophila* cultured cell

INTRODUCTION

Apoptosis is a form of cell death that play a role in development, tissue homeostasis, and disease.[1] Various diseases such as neurodegenerative diseases or cancer evolve because of hyperactivation or suppression of apoptosis.[2] In cancer, the balance between proliferation and apoptosis is disturbed, and defects in apoptotic pathways allow cells with genetic abnormalities to survive. Therefore, the induction of apoptosis is governed by an elaborate array of checks and balances in the cell. A major player in apoptosis is the mitochondrial permeability transition pore (MPTP), a non-specific pore. The MPTP contains hexokinase, the benzodiazepine receptor, cyclophilin D, voltage-dependent anion channel (VDAC) and adenine nucleotide translocase (ANT).[3]

Among the various MPTP component, ANT is the most abundant protein in mitochondria and catalyzes

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the exchange of adenine nucleotides across the mitochondrial inner membrane.[4] In humans, there are three different functional ANT genes encoding proteins with 88~89% amino-acid sequence identity. These show differential tissue expression.[5,6] ANT1 is expressed predominantly in postmitotic cell types in skeletal muscle, heart, and brain; ANT2 is expressed mainly in proliferating tissue types; and ANT3 is expressed ubiquitously.[6,7] They play an important role in normal cell growth and function. In *Drosophila*, two ANT genes showing a high degree of similarity with the mammalian ANT genes have been identified.[8] The *Drosophila* ANT genes are duplicated tandemly, sesB and Ant2 respectively. These two genes are transcribed from a common promoter, and their mRNAs are produced by differential splicing. They are 72% identical in nucleotide sequence and 78% identical in amino acid sequence.[9] Both are seen at all developmental stages and there are clear variations in their levels.[9,10] Although it was reported that ANT expression is decreased with aging and by oxidative stress,[11] the study for function and expression of ANT in *Drosophila* is rarely reported.

The usefulness of the GAL4/UAS system as a tool for targeted gene expression in *Drosophila* has been widely recognized.[12] By combining tissue-specific GAL4 drivers with stably integrated pUAST-based responder constructs, the GAL4/UAS system in specific tissue is an even more precise and powerful tool for functional analysis. In this study, we report that overexpression of *Drosophila* ANT by GAL4-UAS system in *Drosophila* cultured Kc cells caused apoptosis, suggesting that dysregulation of ANT expression may be also associated with apoptosis.

MATERIALS AND METHODS

1) Plasmid construction

To construct pUAST-sesB and pUAST-Ant2, sesB cDNA fragment (1,108 bp) and Ant2 cDNA fragment (1,016 bp) were cloned by polymerase chain reaction (PCR) using *Drosophila* Oregon-R adult cDNA. The used primers, containing the linker sequences 5'-*Not*I and 3'-*Xho*I were as follows: sesB, 5'-GCGGCGGC CGCATGGGCAAGGATT TCGAT-3' and 3'-AGAA TGGTGCTTGTTGCCGAGCTCGCG-5' Ant2, 5'-GC GGCGG CCGCACTAACATGGGCGATGAA-3' and 3'-TCGTTTACAGTGTGGTGCGAGCTCGCG-5'. Then these fragments were inserted into the *Not*I and *Xho*I sites of the pUAST vector. Sequence analysis was performed to confirm the nucleotide sequence.

2) Cell culture and DNA transfection

Drosophila Kc cells were grown at 25°C in M3 (BF) medium (Sigma) supplemented with 2% fetal bovine serum and 0.5% penicillin/streptomycin (Gibco BRL). Transfection of DNA mixtures into Kc cells was performed using dimethyldioctadecyl ammonium bromide (DDAB).[13]

3) RT-PCR

Total RNA from transfected Kc cells was isolated with Trizol Reagent (Molecular Research Center, Inc.) according to the protocol furnished by the manufacturer. The cDNAs was synthesized from 5 μg of total RNA with 200 unit MMLV-RT (reverse transcriptase) (Promega) and 500 ng oligo-dT primer at 42°C for 60min. The PCR mixture contained cDNA derived from 5 μg of total Kc cell RNA, 1.25 unit of *Thermus aquaticus* DNA polymerase (Intron), 200 mM of dNTP, 10×reaction buffer, 10 pmol of each primer. The PCR for sesB or Ant2 cDNA was carried out at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min and 94°C for 1 min, 49°C for 1 min, 72°C for 1 min with 35 cycling, respectively. The RT-PCR products were analyzed on 2% agarose gels stained with ethidium bromide. The PCR product of rp49 was used as control.

4) Acridine orange staining

Acridine orange staining is conducted according to previous report[14] with minor modifications. Transfected Kc cell were incubated for 5 minutes in phosphate-buffered saline (PBS) containing 5 μg/ml of

acridine orange.[14] The cells were placed in fresh PBS and analyzed immediately for nuclear staining on Zeiss Axioskop fluorescence microscope.

RESULTS

1) Amino acid comparisons between the *Drosophila* ANT (SESB and ANT2) and human ANTs (hANT1, hANT2 and hANT3)

We compared amino acid sequences of *Drosophila* two ANTs, SESB and ANT2 with human ANTs from NCBI database by CLUSTAL X[15] (Fig. 1). The amino acid sequence identity of SESB with human ANTs was higher than those of ANT2. The SESB and ANT2 with human ANTs were over 70% identical in amino acid sequence. Therefore, the ANT amino acid sequence was highly conserved between species.

2) Overexpression of *Drosophila* two ANTs, sesB and Ant2 by GAL4-UAS system in *Drosophila* Kc cells

For overexpression of sesB and Ant2 by GAL4-UAS system,[16,17] we constructed the plasmids pUAST-sesB and pUAST-Ant2 as described in Materials and Methods. Expression of sesB and Ant2 in Kc cells transiently transfected with pAc-GAL4/pUAST or pAc-GAL4/pUAST-sesB or pAc-GAL4/pUAST-Ant2 was examined by RT-PCR. The level of sesB mRNA in transfected cells with the plasmids pAc-GAL4/pUAST-sesB was higher than those in transfected cells with the plasmids pAc-GAL4/pUAST and in control cells (Fig. 2A). Also, the expression level of Ant2 in transfected cells with the plasmids pAc-GAL4/pUAST-Ant2 was higher than those in trans-

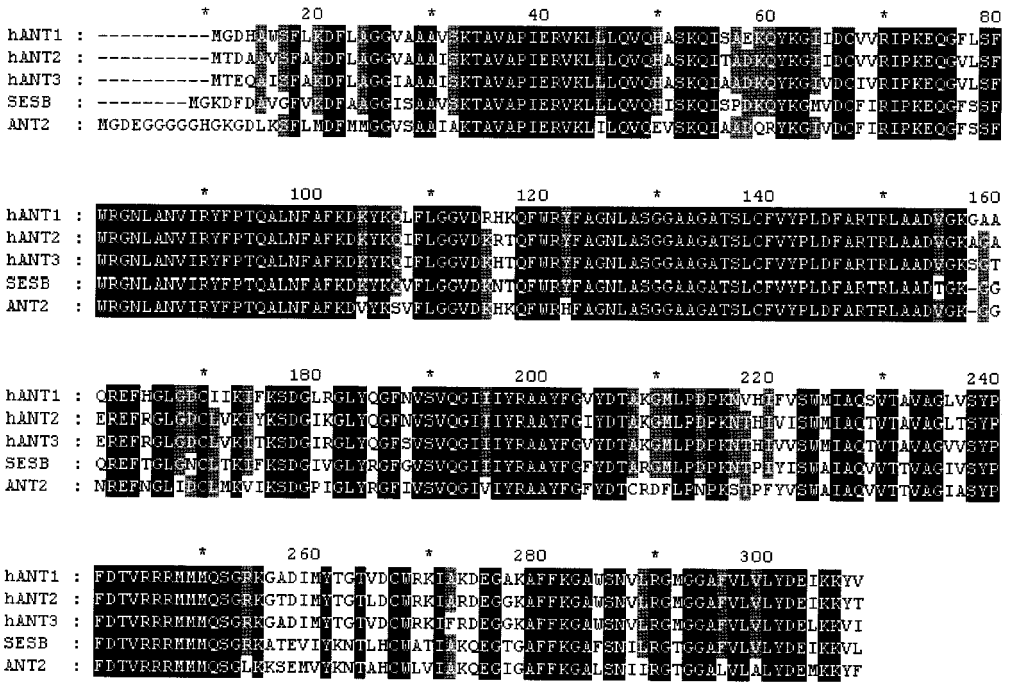

Fig. 1. Amino acid comparisons between the *Drosophila* ANTs (SESB and ANT2) and human ANTs (hANT1, hANT2 and hANT3) by CLUSTAL X.[15] Identical residues are indicated as black or gray box, respectively. All sequences are from NCBI database.

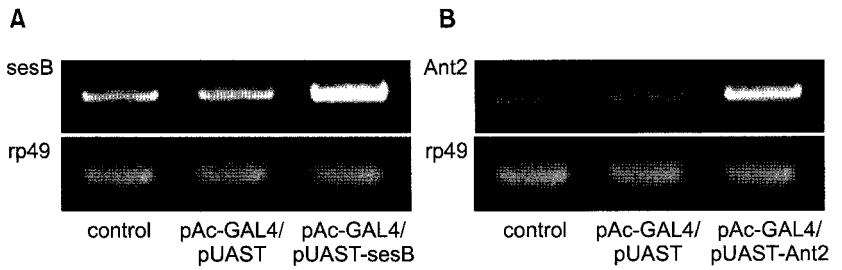

Fig. 2. Overexpression of sesB and Ant2 in *Drosophila* Kc cells by GAL4-UAS system. *Drosophila* Kc cells were transiently transfected with plasmids indicated. After 48 h, Total RNA was prepared from the transfected cells. Then RT-PCR was performed to measure sesB (A) and Ant2 (B) mRNA levels. The ribosomal protein rp49 was used as an internal control. Three independent experiments were performed.

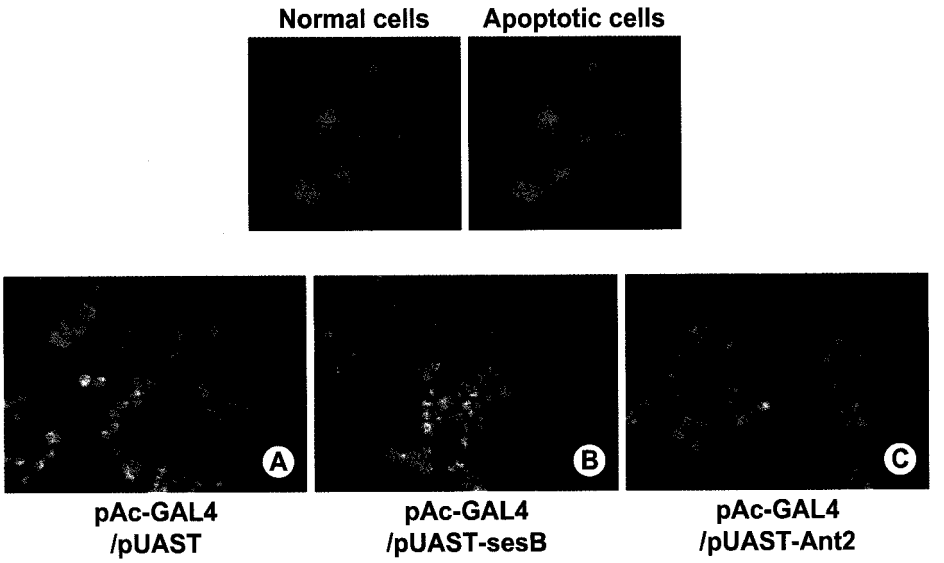

Fig. 3. Overexpression of sesB and Ant2 caused apoptosis in *Drosophila* Kc cell. The pUAST vector (A) or pUAST-sesB (B) or pUAST-Ant2 (C) plasmids were transiently transfected into Kc cells with pAc-GAL4 plasmid. After 48 h, transfected cells were stained with acridine orange to detect apoptotic cells and examined by Zeiss Axioskop fluorescence microscope. In transfected cells with pAc-GAL4/pUAST-sesB (B) or pAc-GAL4/pUAST-Ant2 (C), we observed substantial increased number of apoptotic cells, whereas in transfected cells with pAc-GAL4/pUAST empty vector, a little apoptotic cells were detected (A).

fected cells with the plasmids pAc-GAL4/pUAST and in control cells (Fig. 2B).

3) Overexpression of sesB and Ant2 leads to apoptosis

To investigate the relation between overexpression of *Drosophila* ANT and apoptosis, we transfected Kc cells with the plasmids pAc-GAL4/pUAST, pAc-GAL4/pUAST-sesB or pAc-GAL4/pUAST-Ant2, and then the transfected Kc cells were stained by acridine orange, which is a vital dye that provides a rapid and accurate indicator of apoptosis in insect tissues.[14] Acridine orange is a basic intercalating dye that preferentially labels apoptotic cells[14] and can be used to determine the relative integrity of chromatin.[18] As fragmentation occurs, DNA becomes more accessible

to dye stacking and the concentration of bounded dye particles increases. This increase in dye concentration is seen as a change in the wavelength of light emitted. In these experiments, green nuclear fluorescence indicates low dye concentration and supercoiled DNA, yellow and yellow-orange represents increasing DNA fragmentation and red represents the maximum concentration of dye and highest degree of DNA fragmentation.[18] Transfected cells with pAc-GAL4/pUAST, pAc-GAL4/pUAST-sesB or pAc-GAL4/pUAST-Ant2 revealed variable nuclear staining by acridine orange. In transfected cells with pAc-GAL4/pUAST-sesB (Fig. 3B) or pAc-GAL4/pUAST-Ant2 (Fig. 3C), we observed substantial increased number of apoptotic cells, whereas in transfected cells with pAc-GAL4/pUAST empty vector, a little apoptotic cells were detected (Fig. 3A), The result indicates that overexpression of sesB and Ant2 leads to apoptosis in *Drosophila* Kc cells.

DISCUSSION

The adenine nucleotide translocase (ANT) is a key component of mitochondrial permeability transition pore (MPTP) which plays an important role during apoptosis. In human, it has been reported that hANT1 interact with Bax and is required for apoptosis mediated by Bax.[19] In *Drosophila*, it was reported that expression of ANT is decreased by oxidative stress and with aging.[11] However, function of *Drosophila* ANT is unknown. In the present study, to investigate the function of *Drosophila* ANT and significance of dysregulation of ANT, we constructed the plasmids pUAST-sesB and pUAST-Ant2. Overexpression of *Drosophila* two ANTs, sesB and Ant2 highly conserved with human ANTs, was induced in *Drosophila* cultured Kc cells by GAL4-UAS system. Overexpression of sesB and Ant2 by GAL4-UAS system in *Drosophila* Kc cells transiently transfected with the constructed plasmids was identified by RT-PCR (Fig. 2). Subsequently, we found that overexpression of sesB and Ant2 induces apoptosis in the transfected Kc cells (Fig. 3).

Many genes involved in apoptosis have the dominant capacity to induce cell death upon overexpression. For example, overexpression of reaper, which is a *Drosophila* apoptosis regulatory gene, leads to apoptosis in not only *Drosophila* cell line but also *Drosophila* eyes.[20,21] In yeast cells, Bax can induce a form of cell death by overexpression.[22,23] Furthermore, overexpressed Bax accelerates apoptotic death induced by cytokine deprivation in an IL-3-dependent cell line.[24] Particularly, it was reported that in human, transfection-enforced overexpression of hANT1 induces apoptosis in some cell types, including kidney, ovarian carcinoma or lymphoma cells by transfection.[25] This report is consistent with our results indicating that overexpression of sesB and Ant2 induces apoptosis in *Drosophila* Kc cells. In addition, hANT1 have been known to be specifically and dramatically upregulated in heart tissue of patients with dilated cardiomyopathy (DCM) which is a kind of myocardial.[26] Interestingly, it was reported that excessive apoptosis can be observed in tissues from DCM patients.[27~29] These facts including our results suggest that dysregulation of ANT expression may be associated with apoptosis. To confirm these results *in vitro*, whether ANT overexpression induce apoptosis should be examined *in vivo*.

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초파리 배양세포에서 Adenine Nucleotide Translocase 과발현에 의한 세포사멸유도에 관한 연구

부산대학교 분자생물학과

신 명 주 · 박 소 영 · 유 미 애

Adenine nucleotide translocase (ANT)는 미토콘드리아의 permeability transition pore (MPTP)의 주요한 요소이며, 미토콘드리아 내막 안팎으로 ADP/ATP exchange를 담당한다. 최근 보고에 따르면, 포유동물에서 ANT는 Bax를 매개로 하는 세포사멸에 요구되는 것으로 알려져 있다. 그러나 초파리에서 ANT 기능에 관한 연구는 거의 알려져 있지 않다. 초파리의 ANT 유전자는 염색체상에 이중으로 나란히 배열되어 있으며, 각각 sesB와 Ant2로 명명하고 있다. 본 연구에서는 초파리 유전자의 기능연구에 유용한 시스템인 GAL4-UAS 시스템을 사용하여 초파리 배양 세포에서 sesB와 Ant2의 기능을 조사하였다. 플라스미드 pUAST-sesB와 pUAST-Ant2을 제작하였고, 이들을 초파리 Kc 세포에 pAc-GAL4 플라스미드와 함께 transfection하여 RT-PCR로 sesB와 Ant2의 과발현을 확인하였다. 세포사멸의 정도에 따라 다르게 염색하는 시약인 아크리딘 오렌지(acridine orange)를 사용하여 sesB 또는 Ant2를 과발현시킨 세포와 그렇지 않은 세포를 염색하였다. 그 결과 정상세포에 비해 sesB 또는 Ant2를 과발현시킨 세포에서 세포사멸이 증가함이 관찰되었다. 이러한 결과는 초파리 ANT의 과발현이 세포사멸을 유도함을 나타내며, ANT 유전자의 발현이상이 세포사멸과 관련이 있음을 시사하고 있다.

Key Words: Adenine nucleotide translocase (ANT), 세포사멸, GAL4-UAS 시스템, 초파리 배양세포