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Mcl-1 is a Binding Partner of hNoxa

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The Bcl-2 family proteins play critical roles in regulation of apoptosis, and the balanced interaction of pro- and anti-death members is a key factor in determining the cell fate. Noxa, a BH3-only Bcl-2-family member, has been originally identified as a target gene of p53. To understand the mechanism by which human Noxa (hNoxa) regulates the cell death, we screened the hNoxa binding partner using the yeast two hybrid screening and found that anti-death protein Mcl-1 binds to hNoxa. The binding of hNoxa to Mcl-1 was confirmed by immunoprecipitation in human colon cancer cell line HCT 116 cells. Mcl-1 significantly inhibited the hNoxa-induced cell death in HCT 116 cells. During the cell death induced by hNoxa, Mcl-1 protein was degraded. Its degradation was inhibited by z-VAD-fmk, a pan-caspase inhibitor, suggesting caspase is responsible for Mcl-1 degradation in response to hNoxa. Together, the results indicate that hNoxa binds to Mcl-1 that is degraded by caspases during hNoxa-induced cell death.

Key words – hNoxa, Mcl-1, Bcl-2 family, p53, apoptosis

Introduction

The Bcl-2 family members regulate the cell death by modulating the mitochondrial function, and there are two subgroups, anti-death and pro-death group, depending on the capability of the members that inhibit or promote the cell death, respectively. Noxa, originally identified as a target gene transactivated by tumor suppressor gene p53 [8], belongs to a BH3-only subgroup of pro-death Bcl-2 family members. Mouse Noxa unusually contains two BH3 domains; however, human Noxa (hNoxa) has one BH3 domain like other BH3-only proteins [8,11].

A line of evidence shows a close correlation between p53 level and Noxa induction. p53-dependent cell death is significantly reduced in Noxa-deficient cells, suggesting that Noxa is a crucial player of p53-induced cell death. In addition, it has been shown that hypoxia or proteasome inhibitor Bortezomib (PS-341, Velcade®) can transactivate Noxa in a p53-independent manner [2,6,9,10]. Various deletion mutants of hNoxa demonstrate that hNoxa contains two functional domains, the BH3 domain and mitochondrial targeting domain (MTD). BH3 domain is responsible for the cell death activity of hNoxa, whereas MTD domain is responsible for the translocation of hNoxa to the mitochondria [11,13].

The balanced interaction of anti- and pro-death Bcl-2 family members is a key factor in regulating the cell death. Mcl-1 is an anti-death multidomain Bcl-2 family member that has been shown to bind Bim and Bak on the mitochondrial outer membrane [4,16], and inhibits the leakage of the mitochondrial outer membrane to release the death-promoting proteins such as cytochrome c, Smac/DIABLO, and apoptosis-inducing factor (AIF), resulting in inhibition of the cell death [7]. Mcl-1 also binds to another BH3-only Bcl-2 family member PUMA transactivated by p53, leading to inhibition of the p53-dependent cell death [1].

In this study, to understand the molecular mechanism by which cell death induced by hNoxa is regulated, we have screened the binding partner of hNoxa using yeast two-hybrid system and found that hNoxa binds to Mcl-1 and causes Mcl-1 to be degraded through caspases.

Materials and Methods

Cell culture

Human colorectal cancer cell line HCT 116 was cultured in McCoy'5A medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37℃ and 5% CO_2.

Transfection and Western blotting

Plasmid DNAs were transfected with Effectene trans-

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fection reagent purchased from Qiagen (Chatsorth, CA, USA) according to the company's protocol. Cell lysates were prepared by incubation on ice for 30 min in RIPA buffer (1% NP-40, 0.5% Sodium deoxycholic acid, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl) containing protease inhibitors (aprotinin, pepstatin A, and phenylmethylsulfonyl fluoride), followed by centrifugation of cell lysates at 13,000 *x g* for 15 min and collecting the supernatants. Samples were subjected to 15% SDS-PAGE and immunoblotted with indicated with anti-Mcl-1 antibody (Calbiochem, Darmstadt, Germany), anti-Noxa antibody (Calbiochem, Darmstadt, Germany), and anti-β-actin antibody (Chemicon, Temecula, CA, USA). Bands were visualized with ECL solution.

Immunoprecipitation

Cell lysates prepared as described above were incubated with anti-Mcl-1 antibody and protein-A bead (Pierce, Rockford, IL, USA) in RIPA buffer for 1 hr at 4℃, followed by centrifugation at 500 *x g* for 5 min and collecting pellets by removing the supernatants. The pellets were re-suspended with RIPA buffer, followed by centrifugation and collecting pellets three times as described above for washing out the nonspecific bindings. Upon three washing steps, SDS-PAGE sample loading buffer (200 mM Tris-HCl, 400 mM DTT, 8% SDS, 0.4% bromophenol blue, 40% Glycerol, 2% 2-mercaptoethanol) was added to the pellets for subjecting to 15% SDS-PAGE and western blotting against Mcl-1 and hNoxa.

Cell death assay

For the relative death ratio, the dead cells and survived cells of EGFP positive cells were counted and calculated in percentile.

Yeast two-hybrid screening

Full length of hNoxa cDNA was amplified by PCR using two primers 5′-ccggaattcATGCCTGGGAAGAAGGCGC GC-3′ and 5′-cgaattcTCAGGTTCCTGAGCAGAAGAG-3′, and cloned at *Eco*R1 site of pGBT9 vector. Screening of hNoxa binding partner using rat liver cDNA library in pACT2 vector (Clontech, Mountain View, CA) and pGBT9-hNoxa was performed as the manufacture's protocol.

Results

To identify the binding partner of hNoxa, we adopted the yeast two-hybrid screening method using hNoxa as a bait and rat adult liver cDNA library. It appeared that hNoxa is toxic to yeast cells because only two survived clones were found. The survived clones contained A1 and Mcl-1 which are the members of anti-death Bcl-2 family. The sequencing data of Mcl-1 or A1-containing clone were shown in Fig. 1. We decide to further characterize the functional meaning of the interaction between Mcl-1 and hNoxa.

In order to test whether the binding of hNoxa to Mcl-1 is specific in mammalian cells, human colorectal cancer cell line HCT116 was transfected with pEGFP-hNoxa, and then Mcl-1 was immunoprecipitated with anti-Mcl-1 antibody or only protein-A bead. hNoxa was co-precipitated with Mcl-1 in pEGFP-hNoxa transfected cells but not in non-transfected cells. Mcl-1 bands were detected in Mcl-1 immunoprecipitates from both pEGFP-hNoxa transfected cells and nontransfected cells. No bands of hNoxa and Mcl-1 were observed in immunoprecipitates using protein-A bead. These results suggest that hNoxa specifically binds to Mcl-1 (Fig. 2).

Mcl-1 has been known to inhibit the apoptotic cell death induced by various death stimuli [1,3,7]; thus, we hypothesized that Mcl-1 could inhibit the hNoxa-induced cell death. To examine this hypothesis, HCT116 cells were co-transfected with pEGFP-hNoxa and pcDNA3-Mcl-1. As shown in Fig. 3, in consistent with previous reports, pEGFP-hNoxa caused the significant number of cell death in HCT116 cells compared to pEGFP vector alone, and the cell death induced by hNoxa was inhibited by Mcl-1 in a

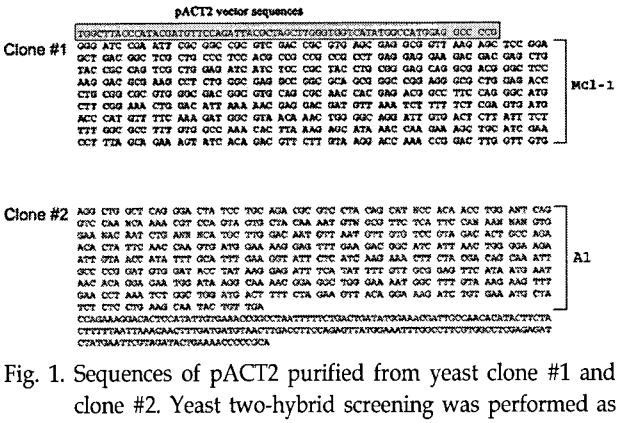

Fig. 1. Sequences of pACT2 purified from yeast clone #1 and clone #2. Yeast two-hybrid screening was performed as described in Materials and Methods. A positive clones were analyzed by DNA sequencing, and identified as the rat Mcl-1 and A1. The shade region indicates the DNA sequences from pACT2, and the regions of Mcl-1 and A1 sequences were indicated.

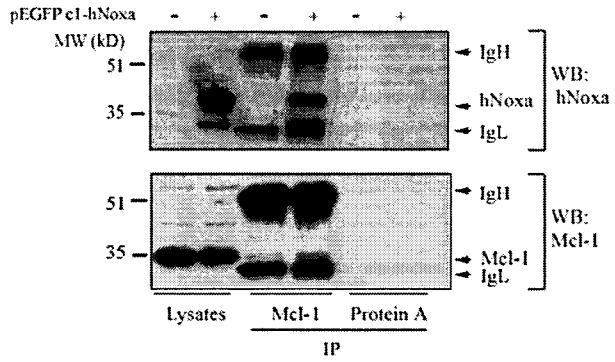

Fig. 2. Mcl-1 binds to hNoxa in mammalian cells. pEGFP-hNoxa plasmid DNA was transfected into HCT116 cells, and lysates were prepared for immunoprecipitation that was performed using anti-Mcl-1 antibody or bead alone. Mcl-1 and hNoxa were visualized by western blot analysis.

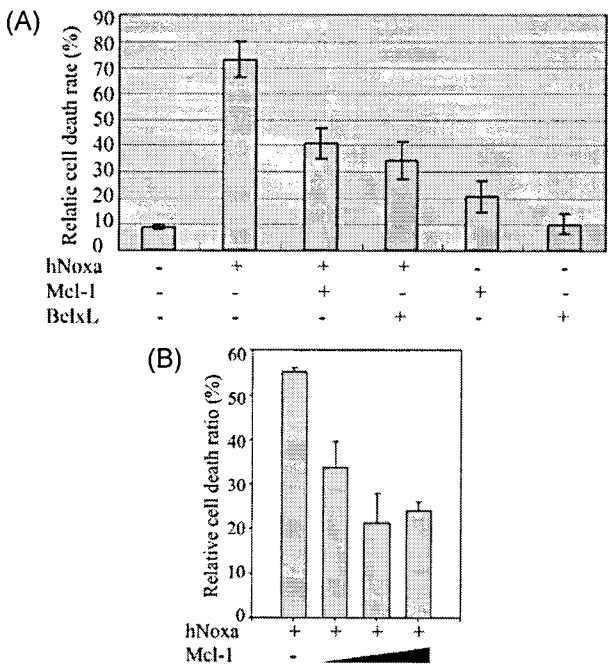

Fig. 3. Mcl-1 inhibits hNoxa-induced cell death in HCT116 cells. (A) HCT116 cells were co-transfected with pEGFP-hNoxa (0.2 μg), pcDNA3-Mcl-1 (0.5 μg), or pcDND3-Bcl-$_{xL}$ (0.5 μg) as indicated. The relative death ratio was determined by counting the dead cells and survival cells of EGFP positive cells. (B) HCT116 cells were transiently transfected with 0.5 μg of pEGFP-Noxa and increasing doses of Mcl-1 (0.2, 0.4, and 0.6 μg). After 30 hr of transfection, relative cell death was counted as the ratio propidium iodide (PI)-positive cells to the EGFP expressing cells.

comparable degree by Bcl-$_{xL}$. Furthermore, to confirm the inhibition of hNoxa-induced cell death by Mcl-1, we co-transfected the different dosage of pcDNA3-Mcl-1 with the constant amount of pEGFP-hNoxa and found that Mcl-1 inhibits the hNoxa-induced cell death in a dose-dependent way. This indicates that Mcl-1 functions as an inhibitor of hNoxa by binding to hNoxa.

Since significant amount of Mcl-1 is endogenously expressed in various types of cells; thus, hNoxa should get rid of the endogenous Mcl-1 protein in order to induce the cell death. Mcl-1 has been known as a very unstable protein due to the PEST sequence at N-terminus of Mcl-1 and also degraded by ubiquitin/proteasome pathway and/or caspases during the cell death [5,12,14,15,17]. Thus, we hypothesized that hNoxa causes the degradation of Mcl-1 by proteasome or caspase, leading to the cell death. To test this hypothesis, HCT116 cells were transfected with pcDNA3-Mcl-1 in the different dosages of pEGFP-hNoxa and assessed the changes of Mcl-1 proteins by immunoblot analysis. As shown in Fig. 4, the amounts of Mcl-1 proteins were decreased when the expression of EGFP-hNoxa was increased. To see whether endogenous Mcl-1 protein is decreased in response to genotoxic agents or not, we monitored the endogenous Mcl-1 proteins during the cell

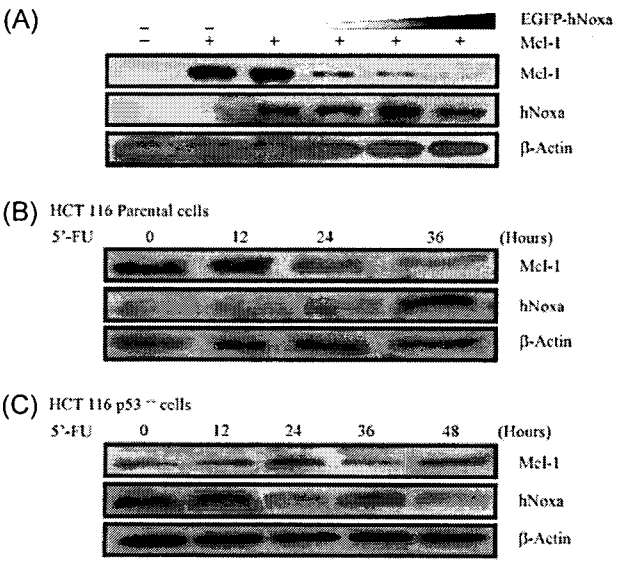

Fig. 4. Mcl-1 is degraded during the cell death induced by hNoxa. (A) HCT116 cells were co-transfected with increasing doses of pEGFP-hNoxa (0, 0.2, 0.4, 0.6, and 0.8 μg) and 0.5 μg of pcDNA3-Mcl-1 for 24 hr. Then, lysates were subjected to western blot analysis using anti-Mcl-1 and anti-hNoxa antibodies. (B and C) At indicated times after 5'FU (200 μg/ml) treatment in HCT 116 parental cells (B) or HCT116 p53$^{-/-}$ cells (C), the cells lysate were separated by 15 % SDS-PAGE. Proteins blotted on PVDF membrane were detected with anti-Mcl-1 antibody and anti-β-actin antibody.

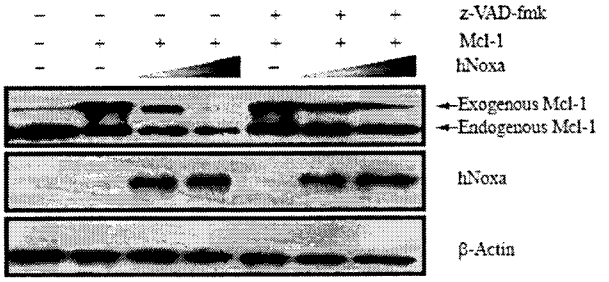

Fig. 5. Degradation of Mcl-1 was inhibited by z-VAD-fmk. HCT116 cells were transiently transfected with 0.5 μg of pcDNA3-Mcl-1 and various dose of pEGFP-hNoxa (0.2, 0.6 μg) with or without z-VAD-fmk (100 μM). Lysate proteins were analyzed by western blotting with anti-Mcl-1 antibody, anti-Noxa antibody, and anti-β-actin antibody.

death induced by 5'-FU that causes the cell death in a p53-dependent manner. Parental HCT116 cells and p53-deficient HCT116 cells were treated with 5'-FU, and monitored the amounts of Mcl-1 protein. Mcl-1 protein was decreased at 24 hr after 5'-FU treatment, and it was evident at 36 hr in parental HCT116 cells; however, Mcl-1 proteins showed no changes in p53-deficient cells. These results implicate that hNoxa causes the degradation of Mcl-1 probably by binding to Mcl-1.

Mcl-1 has been reported to be degraded by ubiquitine/proteasome or caspases during apoptotic cell death [5,12,14, 15,17]. Thus, we hypothesized that hNoxa activates the ubiquitine/proteasome or caspases to degrade Mcl-1 during apoptotic cell death. To investigate this hypothesis, HCT116 cells were transfected with pEGFP-hNoxa and pcDNA3-Mcl-1 in the presence or absence of proteasome inhibitor MG132 or caspase inhibitor zVAD-fmk. Proteasome inhibitor MG132 was very toxic to HCT116 cells so that we could not see the effects of ubiquitine/proteasome. Caspase inhibitor z-VAD-fmk completely blocked the Mcl-1 degradation induced by hNoxa (Fig. 5).

Discussion

In order to understand the molecular mechanism by which hNoxa induces the mitochondrial damage, searching for the binding partner of hNoxa would be the first step so that we have screened the binding partner of hNoxa using a classical yeast two-hybrid system. Two positive colonies that contain the anti-death Bcl-2 family members Mcl-1 and A1 were found, possibly resulting from the inhibitory effects of Mcl-1 and A1 on hNoxa-induced cell death in yeast. Identification of Mcl-1 and A1 as a binding partner of hNoxa is agreement with the reports that Noxa BH3 domain selectively binds to BH3 domains of Mcl-1 and A1 rather than any other BH3 domains of Bcl-2 family members such as Bcl-2 or Bcl-$_{xL}$ [1,7].

The fact that relatively high amounts of Mcl-1 is already expressed in many cell types raises a question about the way how hNoxa induces the cell death even in the presence of Mcl-1. Our results indicated that Mcl-1 is degraded by hNoxa overexpression or genotoxic agent 5'-FU, and Mcl-1 degradation could not be observed in p53-deficient HCT116 cells in response to 5'-FU (Fig. 4). Caspases may be responsible for the degradation of Mcl-1 since caspase inhibitor z-VAD-fmk can block the degradation of Mcl-1 induced by hNoxa overexpression (Fig. 5). Although the results did not specify the responsible caspase for the degradation of Mcl-1 induced by hNoxa, caspase-3 would be the one because recombinant caspase-3, but not caspase-8, has been shown to cleave Mcl-1 protein at Asp 127 and Asp 157 residues [5,15]. Nevertheless, recent reports showed that Mcl-1 is degraded by polyubiquitination and proteasome in response to UV, and Mule (Mcl-1 ubiquitin ligase E3)/ARF-BP1 is responsible for Mcl-1 polyubiquitination [17]. Because we did not exclude the possibility that Mule-mediated Mcl-1 degradation may be involved in our experimental system, Mcl-1 degradation caused by hNoxa could be mediated by proteasome. However, our results prefer that Mcl-1 is more likely mediated by caspases during the cell death induced by hNoxa.

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초록 : Mcl-1 단백질은 Noxa 단백질의 결합 파트너이다.

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Bcl-2 family 단백질은 세포사 조절에 매우 중요한 역할을 하며 세포사 촉진 Bcl-2 family 단백질과 세포사 억제 Bcl-2 family 단백질 사이의 균형적인 상호작용이 세포의 운명을 결정하는 주요인자이다. Bcl-2 family 단백질 중 하나인 Noxa 단백질은 p53에 의한 전사되는 단백질로 처음 발견되었다. Noxa 단백질이 어떻게 세포사를 조절하는지를 이해하기 위해 Yeast two-hybrid 방법을 통해 Noxa 단백질과 결합하는 파트너 단백질을 검색하였고 이를 통해 세포사 억제 단백질 중 하나인 Mcl-1를 발견하였다. 사람 대장암 세포주인 HCT 116에서 Noxa 단백질과 Mcl-1 단백질이 결합하는 것을 면역침전 방법을 통하여 확인하였다. HCT 116 세포주에서 Mcl-1 단백질 과다발현은 Noxa에 의한 세포사 유도를 크게 억제하였다. Noxa 단백질 과다발현에 의한 세포사 과정에서 Mcl-1 단백질이 분해되는 것을 발견하였고 이는 caspase 억제제인 z-VAD-fmk에 의해서 억제되었다. 이는 Mcl-1 단백질이 caspase에 의해서 분해되는 것으로 간주된다. 결론적으로, Noxa와 Mcl-1의 결합은 세포사 과정 중 caspase에 의한 Mcl-1 단백질 분해를 유도를 매개할 수 있을 것으로 추측된다.