Nivalenol induced apoptosis in human peripheral blood lymphocytes \textit{in vitro} and mouse peripheral blood lymphocytes \textit{in vivo}

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Summary

It has been reported that T-2 toxin, a trichothecene mycotoxin produced by \textit{Fusarium} species, induces cellular destruction in bone marrow and thymus cells, followed by reduced numbers of peripheral white blood cells, and this mycotoxin evokes apoptotic cell death in human peripheral blood lymphocytes (hPBLs). To clarify a possibility that the affects of nivalenol (NIV), a trichothecene mycotoxin produced by \textit{Fusarium} species, on peripheral blood cells cause reduction in the number of peripheral blood cells, we analyzed nivalenol-induced apoptosis in hPBLs \textit{in vitro} and mouse PBLs (mPBLs) \textit{in vivo} using flow cytometer. NIV induced apoptosis in hPBLs time-and dose-dependently, while 1, 2-Bis (o-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA-AM), an intracellular Ca++ (Ca) chelator, efficiently inhibited the apoptotic cell death just like T-2 toxin-induced apoptosis.

Key words: nivalenol (ニバルノール), apoptosis (アポトーシス), peripheral blood lymphocytes (末梢血リンパ球), trichothecenes (トリコテセン), flow cytometry (フローサイトメトリー)

Introduction

NIV is one of the trichothecenes, toxic metabolites produced by several fungal genera and isolated from metabolites of \textit{Fusarium} sp. Fn 2B¹⁰. Mechanism of cytotoxic effect of NIV is

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induction of protein syntheses\(^3\). Histopathological analyses of the animals acutely and subacutely exposed to NIV demonstrated that NIV induced karyorrhexis, cellular destruction in bone marrow cells\(^4\). In 1995, it was found that NIV induces apoptotic cell death in HL-60 cells as a new evidence\(^5\). Recently, T-2 toxin induced apoptosis in hPBLs at concentrations of 0.1-100 ng/ml time-and dose-dependently, while BAPTA-AM, a Ca\(^{++}\), chelator, efficiently inhibited the apoptotic cell death\(^6\). In this paper, we studied direct effects of NIV for PBLs in vitro and in vivo using by flow cytometer and Ca\(^{++}\), chelator.

**Materials and Methods**

**Toxin and cell culture**

NIV was isolated and purified from metabolites of *Fusarium sp.* Fn 2B in our laboratory\(^9\). Human peripheral blood lymphocytes (hPBLs) were isolated from healthy volunteers by density-gradient centrifugation using Ficoll-Conary (Immuno-Biological Laboratories, Fujioka, Japan). The hPBLs (5 \times 10^5 cells/ml) were cultured in RPMI 1640 (Nikken Bio Medical Lab, Kyoto, Japan) supplemented with 50 units/ml of penicillin G (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), 50 μg/ml of streptomycin sulfate (Meiji Seika Co. Ltd., Tokyo, Japan), 10% heat-inactivated fetal calf serum (FCS) and 1-1,000 ng/ml of NIV at 37°C in a 5% CO₂ humid atmosphere.

**Flow cytometric apoptosis analysis**

We used a flow cytometric method for measuring the percentage of apoptotic nuclei after propidium iodide (PI) staining. For quantitative analysis of apoptosis, the method was described previously\(^8\).

**Chelation of intracellular calcium ion**

BAPTA-AM (Wako Pure Chemical Industries Ltd., Osaka, Japan) is hydrolyzed by cytosolic esterases and is trapped intracellularly as the active Ca\(^{++}\), chelator BAPTA\(^7\). HPBLs preincubated with 5 μM of BAPTA-AM (50 mM stock solution in dimethyl sulfoxide (DMSO)) for 1 hr were untreated and treated with 1-1,000 ng/ml of NIV at 37°C for 1, 3, and 5 days. The degree of inhibition of apoptosis was analyzed with a flow cytometer as described above. DMSO in a final concentration of 0.05% was employed as a background control.

**Apoptosis analysis in mouse in vivo**

Detection of programmed lymphocyte death is so difficult in finding apoptotic bodies in vivo because of recognition and removal by phagocytes\(^8\), and neither morphological signs of apoptosis nor oligonucleosomal DNA fragmentation were observed among peripheral lymphocytes without previous in vitro culture, even when lymphocytes were isolated ex vivo during the phase of in vivo depletion\(^9\)–\(^13\). In this experiments, apoptosis was also detected by in vitro culture. MPBLs were isolated from ICR mice (5 wks old, female) given i.p. 4.1 mg/kg body weight of NIV or saline by density-gradient centrifugation using Ficoll-Conray. The cells were cultured in medium (RPMI 1640 supplemented with 10% FCS and antibiotics) for 16 and 48 hr at 37°C in a 5% CO₂ humid atmosphere.
Results and Discussion

In the previous experiment, NIV induced a typical apoptosis in HL-60 cells upon the observation of apoptotic bodies under electron microscope and DNA fragmentation on agarose gel electrophoreses. Flow cytometric analyses showed the induction of apoptosis in hPBLs in vitro, as observed in HL-60 cells. DNA fluorescence of PI-stained hPBLs incubated with 1,000 ng/ml of NIV for various times were profiled (Fig. 1). The T-2 toxin-treated hPBLs showed

![Fig. 1 Detection of apoptotic nuclei by flow cytometer. Isolated hPBLs were incubated in the absence (a) or presence of 1,000 ng/ml NIV for 1 (b), 3 (c) and 5 (d) days. The percentages of NIV-induced apoptosis were detected by fluorescence intensity of PI. Apoptotic nuclei appeared as a broad hypodiploid DNA peak which was easily discriminable from the narrow peak of hPBLs with normal DNA content in the red fluorescence channels. PI is excited at 488 nm and the emission conveniently collected around at 630 nm.](image)

![Fig. 2 Percentages of nivalenol-induced apoptosis in vitro. The percentages of apoptotic cells were analyzed by flow cytometer (See Fig. 1). HPBLs were incubated without NIV (△) and with nivalenol at 0.01 ng/ml (▲), 0.1 ng/ml (○), 1 ng/ml (●), 10 ng/ml (□), and 100 ng/ml (■), in RPMI 1640 supplemented with 10% FCS.](image)

![Fig. 3 Suppression of apoptosis by chelating Ca++. HPBLs pretreated with 5 μM BAPTA-AM for 1 hr were incubated without NIV (△) and with NIV at 0.01 ng/ml (▲), 0.1 ng/ml (○), 1 ng/ml (●), 10 ng/ml (□), and 100 ng/ml (■), in RPMI 1640 supplemented with 10% FCS. The percentages of apoptotic cells were determined by flow cytometer (See Fig. 1).](image)
significant reduction of the fraction containing G0/G1 phase nuclei with diploid DNA content, while the apoptotic fraction was greatly increased. These changes were detectable in the cells treated with more than 100 ng/ml of NIV after 1 day. (Fig. 2). In the previous paper, we showed that HL-60 underwent apoptosis by T-2 toxin transiently elevated Ca++i level and the apoptosis was completely inhibited by BAPTA-AM, a Ca++i chelator10 and hPBLs were sufficiently suppressed the T-2 toxin-induced apoptosis6. NIV-treated hPBLs similarly showed suppression of undergoing apoptosis (Fig. 3). The results suggest the possibility that NIV elicits apoptosis in hPBLs like as T-2 toxin-induced hPBLs apoptosis through Ca++i-mediated multiple signal transduction pathways, although the induction potential of NIV is about 100-fold less than T-2 toxin. Next, we demonstrated whether NIV induces apoptosis in PBLs in vivo. By in vitro culture, about 1.5-2 fold apoptotic nuclei were detected in mPBLs isolated from NIV-treated mice in compared with negative controls and the percentages of apoptotic nuclei tended to increase time-dependently within 2 hr (Fig. 4). Moreover, in other tissues such as thymus, spleen and liver apoptotic bodies were observed by electron microscopy10 (see p. 39-43). These in vitro and in vivo observations suggest that NIV-induced cellular damages and cell death are mediated by an induction of apoptosis.

References