

Apoptosis Induced by 4-HPR on Human Stomach Adenocarcinoma Cell Line SNU1*

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—Abstract—

Purpose : Retinoids derived from vitamin A have diverse effects on development, morphogenesis, and homeostasis. They also have effects for prevention and treatment of cancers. In this study, the effect of *N*-(4-hydroxyphenyl) retinamide (4-HPR) on growth and/or proliferation of human gastric adenocarcinoma cell line SNU1 was investigated.

Materials and Methods : The cytotoxic effect of 4-HPR was assessed by MTT assay. The apoptosis induced by 4-HPR was analyzed with cytoplasmic DNA Fragmentation, flow cytometry, and Western blot.

Results : 4-HPR induced cell death of SNU1. The cytoplasmic DNA fragmentation was increased time dependently after treatment of 4-HPR and the cells in the sub-G₀/G₁ fraction of flow cytometric analysis were also increased time dependently after treatment of 4-HPR. The cleavage of caspase 3 and PARP were detected after treatment of 4-HPR to SNU1.

The phosphorylations of Raf, ERK and AKT were induced by 4-HPR but after pre-treatment of MAPK inhibitor (PD98059) or PI-3 kinase inhibitor (LY294002) the 4-HPR-induced cytoplasmic DNA fragmentation, the cells in the sub-G₀/G₁ fraction fraction of flow cytometric analysis, and cleavage of caspase 3 and PARP were diminished in SNU1 cells.

Conclusion : The results show that 4-HPR induces apoptosis of SNU1 and this 4-HPR-induced apoptosis may be mediated through ERK1/2 and PI3 kinase signaling pathways in SNU1.

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Key Words: 4-HPR, SNU1, apoptosis.

Introduction

Retinoids, such as vitamin A and its biological derivatives and synthetic analogs, have diverse effects on development, morphogenesis, and homeostasis.¹⁻³⁾ They have been tested for the prevention and treatment of cancers.^{4,5)} Natural retinoids show limited effects on many cancer cells. However, several synthetic retinoids exhibit potent biological activities.⁶⁾

The synthetic retinoid, *N*-(4-hydroxyphenyl) retinamide (4-HPR) prevents chemically induced cancers of the breast, prostate, bladder and skin in animal model.⁷⁻¹⁰⁾ In clinical trials, complete or partial remission did not occur in advanced breast cancer patients treated with 4-HPR.¹¹⁾ But, 4-HPR have more pronounced effect on treatment of breast cancer combined with tamoxifen,¹²⁾ treatment of early stage prostate cancer,¹³⁾ and lowering the secondary tumor formation in patients with breast, head and neck, and prostate cancers.¹⁴⁻¹⁶⁾ Thus, preclinical/clinical data show promise of usefulness of 4-HPR as a chemopreventive agent.

Apoptosis, now widely recognized as a common mechanism of physiological cell deletion, is a distinctive and active mode of cell death with characteristic morphological and biochemical features such as chromatin

condensation, nuclear disintegration, and cellular fragmentation into clusters of eosinophilic globules. Apoptosis can be confirmed by several experimental methods such as flow cytometry, DNA ladder formation, TUNEL assay, and PARP cleavage.¹⁷⁾

The mechanisms by which 4-HPR induces apoptosis can be explained several ways. The endogenous ceramide levels were transiently increased by treatment with 4-HPR in HL-60 cells. And fumonisin B1, which prevents *de novo* biosynthesis of ceramide, blocked the increase in ceramide levels and prevented apoptosis in HL-60 cells.¹⁸⁾ Reactive oxygen species (ROS), *e.g.*, superoxide and H₂O₂, are important mediators of apoptosis. 4-HPR treatment induces an elevation in ROS in a number of cell types. The relative amounts of ROS generated are cell-type-dependent and also varies with a dose of 4-HPR used.¹⁹⁾ Pretreatment of cells with antioxidants prevents the elevation of ROS and effectively inhibits apoptosis. Addition of catalase also protects against cell death, further supporting a role for ROS in mediating 4-HPR-induced apoptosis.²⁰⁾ HPR was also reported to sustain c-Jun N-terminal kinase (JNK) activation in LNCaP cells, by a caspase-independent mechanism.²¹⁾

The ability of retinoic acid to induce apoptosis in gastric cancer cell was investigated in the

human gastric cancer cell lines MKN-45 and MKN-28.²²⁾ Liu et al.²³⁾ reported that 4-HPR induced apoptosis in stomach adenocarcinoma cell line BGC823, at least in part via the retinoic acid receptor (RAR) β . But signal mechanisms of 4-HPR induced apoptosis in human gastric cancers are still uncertain.

In this study, the effect of 4-HPR on growth and/or proliferation of human gastric adenocarcinoma cell line SNU1 was investigated. It was defined that the 4-HPR induced cell death was mediated by apoptosis or necrosis. And it was investigated which signals were involved in 4-HPR-induced apoptosis.

Materials and Methods

1) Cell culture and 4-HPR treatment

Human stomach adenocarcinoma cell line SNU1 was purchased from Korean Cell Line Bank (KCLB, Seoul, Korea). SNU1 cells were maintained in RPMI1640 supplemented with 10% FBS and 100 μ g/ml antibiotic solution at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

Cells were grown in 6 cm diameter culture dishes starting at an initial density of 5×10^5 cells/3 ml medium/dish. After incubation for 24 h, cells were starved in RPMI1640 + 1% FBS for 48 h, and then treated with 4-HPR. LY 294002 (20 μ M) or PD 98059 (20 μ M) was treated 1 h prior to treatment of 4-HPR. Media were changed

with RPMI1640 + 1% FBS and 4-HPR every other day.

2) MTT assay

The cytotoxic effect was assessed by MTT assay. Cells were seeded at a density of 5×10^3 cells/well in a 96-well microplate and allowed to attach overnight. The next day the cells were treated with different concentrations of 4-HPR. After 70 h, 50 μ l of MTT (50 mg/ml) solution was added to the wells and incubated for 2 h at 37°C. The MTT solution was discarded by aspiration and the resulting formazan products converted by the viable cells were dissolved in 250 μ l of DMSO. The absorbance at 570 nm was measured with a BioRad M450 microplate reader. Cell death was expressed as a percentage of untreated control.

3) Western blot analysis

Cells were lysed with a lysis buffer (50 mM Tris-HCl pH8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 1 mM sodium vanadate and 5 mM NaF) and centrifuged at 15,000 rpm, 4°C for 10 min. Proteins (50 μ g) were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were soaked in 5% nonfat dried milk in TTBS (10 mM Tris-HCl pH7.5, 150 mM NaCl, and 0.05% Tween-20) for 30 min and then incubated overnight with primary antibodies such as anti-phosphoRAS, anti-

phosphoMEK, anti-phosphoERK, anti-phospho PDK, anti-phosphoAKT (Cell Signaling technology, USA), anti-phosphoJNK, and anti-poly(ADP-ribose) polymerase (PARP) (Santa Cruz Biotechnology, CA, USA) at 4°C. After washing three times with TTBS for 10 min, membranes were incubated with a horse-raddish peroxidase-conjugated secondary antibody for 4 h at 4°C. The membranes were rinsed three times with TTBS for 10 min and antigen-antibody complex was detected using the enhanced chemiluminescence (ECL) solution.

4) Flow Cytometry

Cells (5×10^5) were treated with 10 μ M of 4-HPR and harvested at 0, 24, 48, and 72 h after treatment. Cells were fixed with ethanol, and stored at 4°C for 1 h. Cells were rinsed two times rapidly with phosphate buffered saline (PBS) containing 2.5 mM Ca^{2+} . Cells were suspended with 200 μ l of propidium iodide (50 μ g/ml in 1.12% sodium citrate). After incubation at 37°C for 30 min, cells were added with 200 μ l RNase A (50 μ g/ml in 1.12% Sodium citrate) and were incubated at 37°C for 20 min. Propidium-bound DNAs were measured using a FACS scan flow cytometer (Becton Dickinson, San Jose, CA). The sub- G_0/G_1 fraction was obtained by gating hypodiploid cells in the DNA histogram.

5) Analysis of cytoplasmic DNA Fragmentation

Cells (1×10^6) were seeded in 6 cm culture dishes and incubated for 24 h. Cells were treated with 4-HPR for 0, 24, 48, and 72 h. For analysis of cytoplasmic DNA, attached cells and floating cells in media were collected together and lysed with 0.5 ml of lysis buffer (5 mM Tris-HCl, pH 7.4, 20 mM EDTA, 0.5% Triton X-100) containing 0.1 mg/ml RNase A. After incubation at 37°C for 30 min, the cell lysates were treated with 1 mg/ml proteinase K for 16 h at 37°C. DNA was extracted with phenol/chloroform and chloroform, and then was precipitated with 70% ethanol and 0.2 volume of 3 M sodium acetate, pH 8. DNA was dissolved in 50 μ l of H_2O , separated on a 2% agarose gel containing 0.5 μ g/ml ethidium bromide, and visualized using a UV transilluminator.

Results

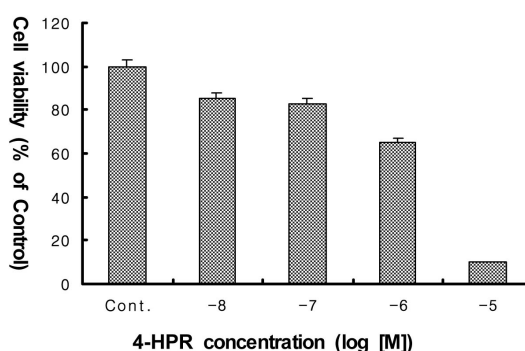


Fig. 1. Effect of 4-HPR on cytotoxicity in SNU1 cells. Cells were treated with increasing concentrations of 4-HPR for 72 h and cytotoxicity was measured by MTT assay. Values are mean \pm SD of triplicates of five independent experiment.

1) 4-HPR-induced cytotoxicity in SNU1 cell

The cytotoxic effect of 4-HPR on SNU1 was analyzed by the MTT assay (Fig. 1). The cytotoxicity induced by 4-HPR in SNU1 was increased in a dose-dependent manner. Complete cell death was observed in cells treated with 10 μ M 4-HPR.

2) 4-HPR-induced apoptosis.

To investigate whether the 4-HPR-induced cell death is mediated by apoptosis or necrosis, cytoplasmic DNA fragmentations and DNA contents in 4-HPR-treated cells were analyzed. Cytoplasmic DNA fragmentation was clearly shown in cells treated with 4-HPR for 48 h and increased in a time-dependent manner (Fig. 2). DNA contents in 4-HPR treated SNU1 cells were stained with

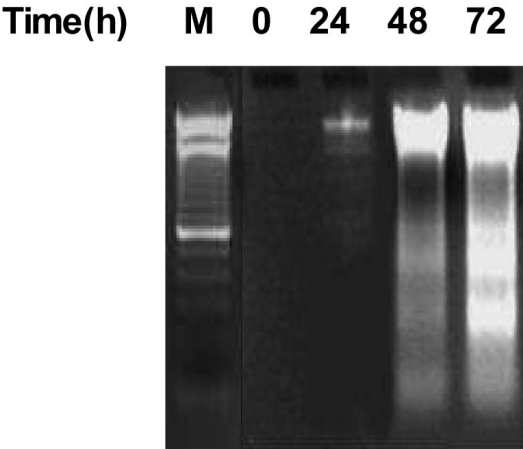


Fig. 2. 4-HPR effect on cytoplasmic DNA fragmentation of SNU1 cell. Cells were treated with 10 μ M of 4-HPR for the indicated time. Cytoplasmic DNA was extracted from attached and floating cells and resolved on a 2% agarose gel.

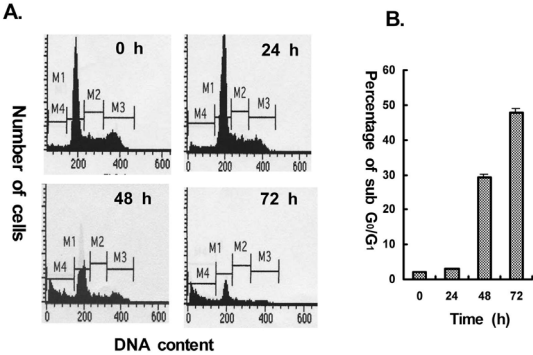


Fig. 3. Flowcytometric analysis of 4-HPR-treated SNU1 cells. Cells were treated with 10 μ M concentrations of 4-HPR for the indicated times. A. DNA contents in the cells were stained with propidium iodide and analyzed by flowcytometry. B. The percentage of sub-G₀/G₁ fraction was measured from three independent experiments. Values are mean \pm SD.

propidium iodide and analyzed by a flow cytometer. The sub-G₀/G₁ fraction was gradually increased in 4-HPR-treated cells with increasing times; 3.03% at 24 h, 29.13% at 48 h and 47.73% at 72 h (Fig. 3).

To further confirm whether the 4-HPR-mediated cell death in apoptosis, cleavages of

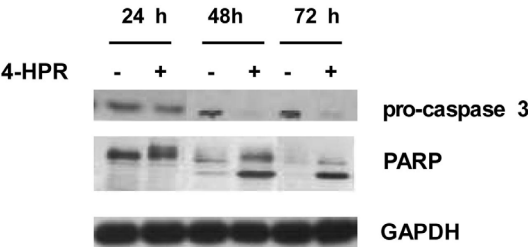


Fig. 4. Effect of 4-HPR on cleavage of caspase 3 and PARP. SNU1 cells were treated with 10 μ M 4-HPR for indicated times. The caspase 3 and PARP cleavage was measured by Western blotting with anti-caspase 3 and anti-PARP antibody.

caspase 3 and PARP, well known to be observed in apoptotic cells, were measured by Western blot analysis (Fig. 4). Decreases in the amount of caspase 3 due to proteolytic cleavage were observed in 4-HPR treated cells in a time dependent manner. PARP cleavages were also increased in 4-HPR treated cells with increasing times. These results suggest that the 4-HPR mediated cytotoxicity in SNU1 cells may be attributed to apoptosis.

3) Effects of 4-HPR on the phosphorylation of MAP kinases and PI 3 kinase.

In order to investigate the signal transduction pathways through which the 4-HPR induced apoptosis in SNU1 cells were mediated,

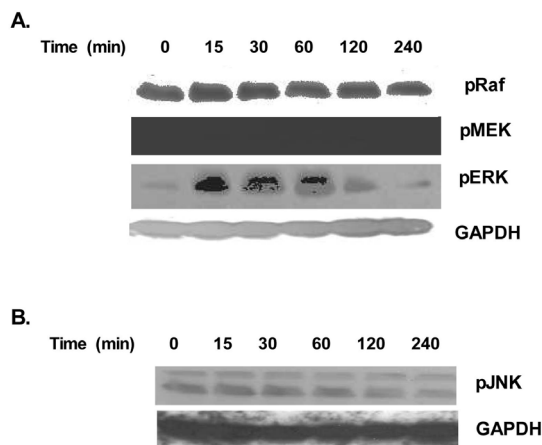


Fig. 5. Effect of 4-HPR on the phosphorylation of mitogen-activated protein kinases. SNU1 cells were treated with 4HPR (10 μ M) for the indicated times. Activations of ERK and JNK were assessed by Western blotting with anti-phosphorylation-specific ERK and JNK antibodies. Equal loading of protein was confirmed by a GAPDH antibody.

activations of MAP kinases including ERK1/2, JNK and p38 were analyzed. Cells treated with 4-HPR showed an increased ERK1/2 phosphorylation compared to untreated cells. 4-HPR induced ERK1/2 phosphorylation was observed in 15 min, sustained to 60 min and gradually decreased in 120 min (Fig. 5A). Upstream signaling molecules of ERK1/2 such as Raf and MEK were also activated. Activations of JNK and p38 were not detected (Fig. 5B). Activation of PI3 kinase was also observed in 4-HPR-treated cells. Downstream signaling molecules activated by PI3 kinase, including PDK and AKT were phosphorylated. PDK phosphorylation was observed in 5 min and sustained to 120 min and AKT phosphorylation was observed in 15 min and sustained to 60 min and gradually decreased in 120 min (Fig. 1).

4) Effects of MAPK inhibition and/or PI3 Kinase inhibition on 4-HPR induced apoptosis.

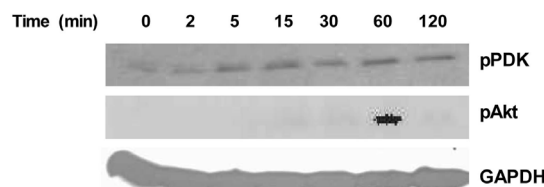


Fig. 6. Phosphorylation of Akt and PDK by 4-HPR. Cells were treated for various times with 10 μ M concentration of 4-HPR. Phosphorylations of Akt and PDK were measured by Western blotting with anti-phospho-Akt and PDK antibodies. Equal loading of protein were estimated with GAPDH antibody.

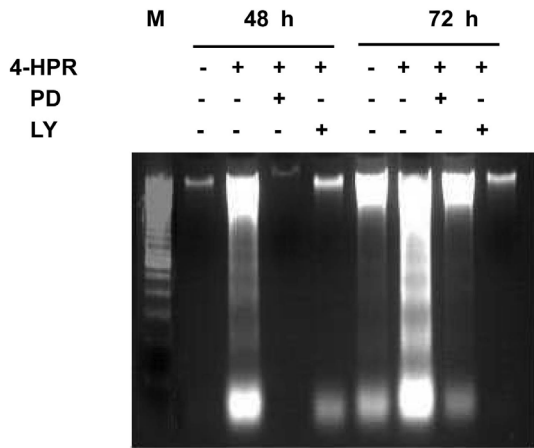


Fig. 7. Effects of PD or LY on 4-HPR induced cytoplasmic DNA fragmentation of SNU1 cell. 4-HPR-induced apoptosis was measured by cytoplasmic DNA fragmentation. SNU1 cells were pretreated with either 20 μ M PD 98059 (PD) or 20 μ M LY 294002 (LY) for 1 h and then treated with or without 10 μ M 4-HPR for the indicated times. Cytoplasmic DNAs were separated on a 2% agarose gel containing ethidium bromide. M, DNA size marker.

To investigate whether the 4-HPR-induced apoptosis was mediated through ERK and AKT phosphorylation, effects of specific inhibitor to ERK (PD 98059) and PI3 kinase (LY 294002) were measured. Pretreatment of either PD 98059 or LY 294002 blocked the 4-HPR induced cytoplasm DNA fragmentation (Fig. 7) as well as cleavages of caspase 3 and PARP. These results suggest that the 4-HPR-induced apoptosis in SNU1 cells is mediated by the activation of ERK and PI3 Kinase.

Discussion

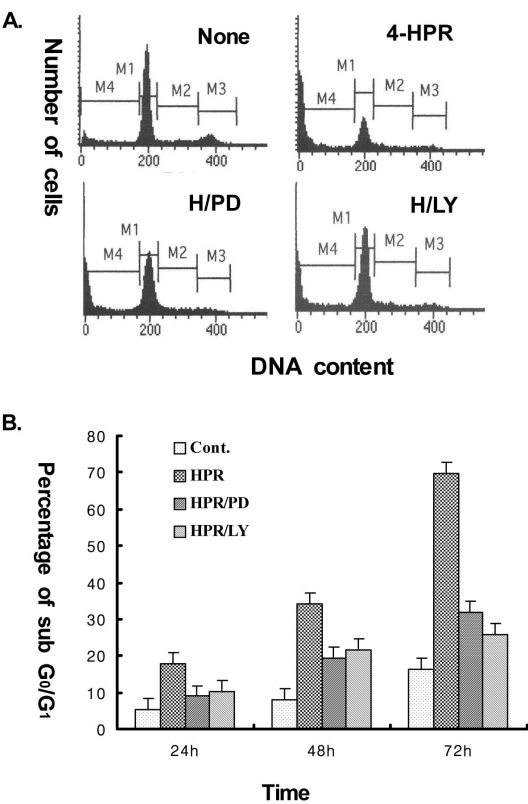


Fig. 8. Flowcytometric analysis of LY or PD effect on 4-HPR-induced apoptosis. SNU1 cells were pretreated with 20 μ M PD 98059 (PD) or 20 μ M LY 294002 (LY) for 1 h and then treated with or without 10 μ M 4-HPR for the indicated times. A. Cells were treated with 4-HPR for 72 h. DNA contents in the cells were stained with propidium iodide and analyzed by flowcytometry. B. The percentage of sub-G₀/G₁ fraction was measured from three independent experiments. Values are mean \pm SD.

4-HPR-induced cell death was increased in a dose-dependent manner in SNU1 cells. Complete cell death was observed at 10 μ M of 4-HPR. The concentrations of 4-HPR which induces apoptosis in a variety of cancer cells are vary from 0.1 to 100 μ M.²⁴⁾ Especially,

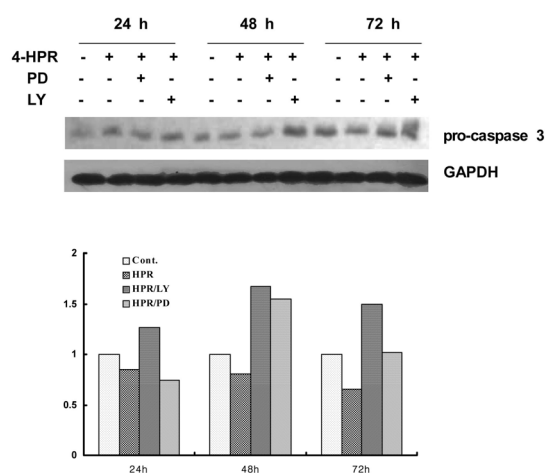


Fig. 9. Effect of LY and PD on 4-HPR induced cleavage of caspase 3. SNU1 cells were pretreated with 20 μ M PD 98059 (PD) or 20 μ M LY 294002 (LY) for 1 h and then treated with or without 10 μ M 4-HPR for the indicated times. The caspase 3 cleavage was measured by Western blotting with a caspase 3 antibody.

stomach adenocarcinoma cell line BGC823 showed apoptosis by treatment with 10 μ M 4-HPR²³⁾, similar to the present study. However, BGC823 cells were treated for a longer time (7 days) than SNU1 cells (3 days), suggesting that SNU1 cells may be more sensitive to 4-HPR than BGC823 cells.

Apoptosis is characterized by the generation of fragmented nuclei with highly condensed chromatin, protrusion of cytoplasm, and formation of apoptotic bodies. And necrosis is defined as cells having electron-lucent cytoplasm and mitochondrial swelling with apparently intact nuclei.¹⁷⁾

The present study showed the increases in cytoplasmic DNA fragmentation, sub-G0/G1

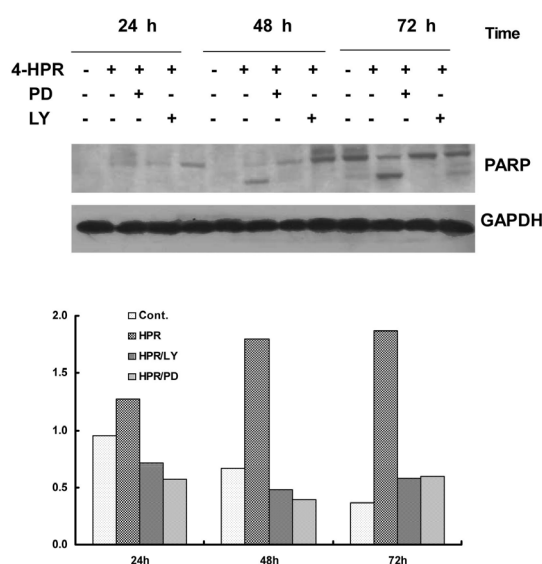


Fig. 10. Effect of LY and PD on 4-HPR induced cleavage of PARP. SNU1 cells were pretreated with 20 μ M PD 98059 (PD) or 20 μ M LY 294002 (LY) for 1 h and then treated with or without 10 μ M 4-HPR for the indicated times. The PARP cleavage was measured by Western blotting with a PARP antibody.

fraction, and cleavages of caspase3 and PARP in 4-HPR-treated cells. These results suggest that the 4-HPR mediated cytotoxicity in SNU1 cells may be attributed to apoptosis.

There was only one report mentioned about the 4-HPR induced-apoptosis in gastric cancer. Liu et al.²³⁾ showed that RAR α receptor is involved in 4-HPR induced-apoptotic signal. However, in other cancer cells, several signals such as ceramide, ROS, and JNK have been reported to be related to 4-HPR induced-apoptosis.^{18, 20, 21)}

In this study, treatment of SNU1 cells with 4-HPR increased in ERK1/2 phosphorylation

and PDK and AKT phosphorylation, suggesting that ERK1/2 and PI3 kinase signal pathways might involve in 4-HPR-induced apoptosis.

The reports of Voelkel-Johnson et al.²⁵⁾ and Widmann et al.²⁶⁾ may explain the increased activation of ERK1/2 and AKT by 4-HPR even though they used different ligand and cells. They showed that several important protein kinases, such as protein kinase C (PKC), the MAP/ERK kinase kinase-1 (MEKK1), Raf-1, Akt-1, Wee-1, and c-src were implicated in the apoptotic pathway in tumor necrosis factor-induced apoptosis in fibroblast and genotoxin-induced apoptosis in human embryonic kidney 293 cell.

By the specific inhibitor to ERK (PD 98059) and PI3 kinase (LY 294002), 4-HPR induced cytoplasm DNA fragmentation and the cleavages of caspase 3 and PARP were diminished in SNU1. These data suggest that the 4-HPR-induced apoptosis is mediated by the activation of ERK and PI3 kinase.

In conclusion, the present study shows that 4-HPR induces growth inhibition in the stomach adenocarcinoma cell line SNU1. 4-HPR-induced cell death is mediated by apoptosis through increased activation of ERK1/2 and AKT.

요 약

사람 위암세포주 SNU1의 4-HPR에 의한 세포사멸사 기전

목적 : Retinoids는 Vitamin A로부터 유도된 물질들로 세포의 증식과 분화, 세포사멸 등을 조절한다고 알려져 있다. 최근 retinoid가 여러 실험 동물에서 암을 억제한다고 알려지고 있다. 본 연구에서는 합성 retinoid인 *N*-(4-hydroxyphenyl) retinamide(4-HPR) 세포사멸의 유도와 세포사멸 관련 세포신호전달 기전을 밝히고자 하였다.

재료 및 방법 : MTT assay, Western blot, flow cytometry, cytoplasmic DNA fragmentation 등의 방법을 사용하여 위암 세포주 SNU1에서 4-HPR의 세포사멸 유도를 관찰하였다.

결과 : 4-HPR가 농도 의존적으로 SNU1에서 세포독성을 나타내었다. Cytoplasmic DNA fragmentation이 4-HPR 처리 후 48시간부터 관찰되었다. Flow cytometric analysis에서 sub-G₀/G₁ 부분의 세포들의 증가와 Western blot에서 PARP와 caspase-3의 단백질 분해의 증가 역시 48시간부터 나타났다. 이러한 결과들이 4-HPR의 SNU1에 대한 세포독성이 세포사멸에 의한 것임을 시사한다. 4-HPR에 의해 유도된 세포사멸 신호 전달 경로를 밝히기 위하여 4-HPR를 처리한 SNU1세포에서 MAP kinase (ERK)와 AKT의 인산화가 증가함을 확인하였다. ERK 억제제 PD98059 또는 PI3 Kinase 억제제 LY294002를 전처리 하였을 때 4-HPR에 의하여 유도된 cytoplasmic DNA fragmentation의 증가와 PARP의 단백질 분해, caspase-3의 단백질 분해가 억제됨을 확인할 수 있었다.

결론 : 이상의 결과에서 사람의 위암 세포주 SNU1에서는 4-HPR에 의한 세포사멸이 ERK와 AKT 세포신호전달과정을 경유한다.

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