



Preliminary report

Fulvic acid promotes extracellular anti-cancer mediators from RAW 264.7 cells, causing to cancer cell death *in vitro*



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ABSTRACT

Fulvic acid (FA) is known to promote electrochemical balance as a donor or a receptor possessing many biomedical functions. Nevertheless, the effect of FA on the anti-cancer activity has not been elucidated. In the current study, we first isolated FA from humus and investigated whether FA regulates immune-stimulating functions, such as production of nitric oxide (NO), in RAW 264.7 cells. Our data showed that FA slightly enhances cell viability in a dose-dependent manner and secretion of NO from RAW 264.7 cells. It upregulated the protein and mRNA expression of inducible NO synthesis (iNOS). In addition, FA enhanced the DNA-binding activity of nuclear factor- κ B (NF- κ B) in RAW 264.7 cells; the NF- κ B inhibitor, pyrrolidine dithiocarbamate (PDTC) effectively attenuated the expression of FA-stimulated iNOS, suggesting that FA stimulates NF- κ B to promote iNOS and NO production. Finally, FA-stimulated culture media (FA-CM) from RAW 264.7 cells were collected and MCA-102 fibrosarcoma cells were cultured in this media. The FA-CM augmented MCA-102 fibrosarcoma cell apoptosis; however, an NO inhibitor *N*^G-monomethyl-L-arginine (NMMA) slightly inhibited the FA-CM-mediated MCA-102 fibrosarcoma cell apoptosis, which was accompanied by low levels of NO. In the present study, we found that FA induces the generation of NO and iNOS in RAW 264.7 cells by inducing NF- κ B activation; however, NO did not significantly stimulate MCA-102 fibrosarcoma cell apoptosis in the current study. In addition, FA-CM enhanced cell death in various human cancer cells such as Hep3B, LNCaP, and HL60. Taken together, FA most likely stimulates immune-modulating molecules such as NO and induces cancer cell apoptosis.

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1. Introduction

Macrophages are the major mononuclear phagocytic cells that release a broad spectrum of immune-regulating mediators including pro-inflammatory substances, cytotoxic molecules, bioactive lipids, hydrolytic enzymes, and growth factors [1]. In response to various immune-regulating cytokines and even chemical compounds, macrophages are activated and they transcriptionally express the inducible nitric oxide synthase (iNOS), resulting in the acceleration of nitric oxide (NO) production [2]. Recent studies have demonstrated that NO-releasing prodrugs enhance pro-apoptotic effects in cancer cells by deregulating cellular redox systems [3,4]. Therefore, compounds that stimulate NO production in the immune system without any cytotoxicity show enhanced anti-cancer effects by inducing apoptosis [5].

Nuclear factor- κ B (NF- κ B) is an inducible transcription factor that regulates the expression of many genes involved in inflammatory and

immune responses [6]. The expression of many inflammatory genes, including iNOS, is modulated by the binding of NF- κ B [6]. NF- κ B is maintained in its inactive form in the cytoplasm owing its coupling with inhibitory molecules. Upon activation of NF- κ B, the large multi-subunit I κ B kinase (IKK) complex phosphorylates the two NH₂-terminal serine residues of I κ B, which is followed by phosphorylation- and ubiquitin-dependent degradation of I κ B [7]. Consequently, the NF- κ B complex translocates to the nucleus and induces the transcription of its target genes such as iNOS [8].

There has been rapidly growing interest in fulvic acid (FA) among the scientific and medical communities. Previous studies have shown that FA has tremendous potential for use, for example, as a powerful organic electrolyte and a nutrient, it promotes enzyme reactions and cell division, and increases metabolism [9,10]. According to Schepetkin et al., FA derived from semihard black mummie increases the production of reactive oxygen species (ROS) and NO in peritoneal macrophages, depending on the content of carbohydrates [11]. In contrast, Wang et al. reported that FAs derived from different origins show significantly dissimilar functions with respect to ROS generation [12]. Nevertheless, the anti-cancer effect of FA has not been reported thus far.

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In the present study, we investigated NO production induced by FA in RAW 264.7 cells. We found that culture medium of FA-stimulated RAW 264.7 cells (FA-CM) induces apoptosis of MCA-102 fibrosarcoma cells and various human cancer cells such as Hep3B, LNCaP and HL60 cells.

2. Materials and methods

2.1. Preparation of FA

Extraction of FA was conducted from Humus by base-acid extraction methods (Fig. 1). Briefly, humus was extracted by treating with 0.1 M NaOH alkaline solution, filtering, and removing insoluble humic acid. Then, the residual solution was acidified to pH 1–2 using 6.0 M HCl to yield FA. The precipitated FA was centrifuged to produce FA fractions at $4500 \times g$ for 30 min. The lyophilized FA was used in this study. Endotoxin contamination was analyzed using a *Limulus* Amebocytes Lysate (LAL) pyrogen kit (BioWhittaker, Walkersville, MD) according to the manufacturer's protocol. Briefly, 100 μ l standard solution, FA, and controls were mixed with same volumes of LAL reagent and incubated for 1 h at 37 °C. The endotoxin concentration of each sample was analyzed at 545 nm. The quantity of endotoxin in FA was ≤ 0.01 ng/mg.

2.2. Reagents and antibodies

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and polymyxin B (PB) were obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-human antibodies against iNOS, p50, p65, C-23, I κ B α , and phospho (p)-I κ B α were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against caspase-3, poly ADP ribose polymerase (PARP), and β -actin were obtained from Cell Signaling (Beverly, MA). Peroxidase-labeled goat anti-rabbit antibody was purchased from KOMA Biotechnology (Seoul, Republic of Korea). Pyrrolidone dithiocarbamate (PDTC) was obtained from Calbiochem (San Diego, CA). RPMI 1640, fetal bovine serum (FBS), and penicillin-streptomycin mixture were purchased from WelGENE Inc. (Daegu, Republic of Korea). Other chemicals were purchased as Sigma grades.

2.3. Cell culture and viability

RAW 264.7 cells, MCA-102 fibrosarcoma cells, human hepatocellular carcinoma Hep3B cells, human prostate carcinoma LNCaP cells, and human leukemia HL60 cells were obtained from American Type Culture

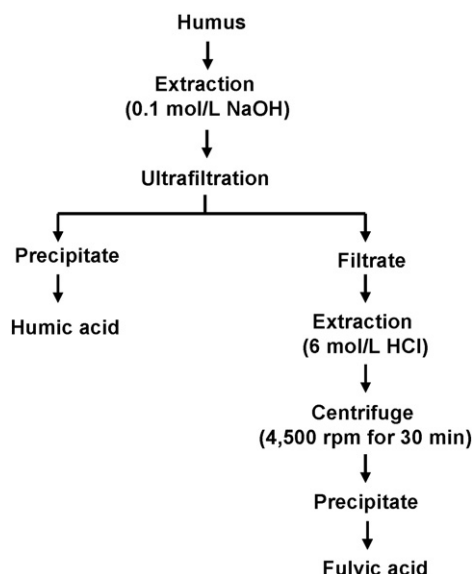


Fig. 1. Isolation procedure of FA.

Collection (Manassas, VA) and cultured at 37 °C in 5% CO₂ containing DMEM supplemented with 5% FBS and 1% penicillin-streptomycin. For the analysis of RAW 264.7 cell viability, the cells were incubated with the indicated concentrations of FA for 24 h. In a parallel experiment, FA-CM was collected at 24 h and treated in various cancer cells for 24 h after dilution with fresh media. Cell viability was determined by an MTT assay and under microscope.

2.4. DNA fragmentation assay

MCA-102 fibrosarcoma cells were treated with FA-CM for 24 h and lysed in DNA fragmentation lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100 for 1 h on ice. Fragmented DNA in the supernatant was isolated with equal volume of phenol:chloroform:isoamyl alcohol mixture and analyzed electrophoretically on 1.5% agarose gel.

2.5. Flow cytometric analysis

MCA-102 fibrosarcoma cells were fixed 1 U/ml RNase A (DNase free) and 10 μ g/ml propidium iodide (Sigma) 1 h at room temperature in the dark conditions. A FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) was used to analyze the level of apoptotic cells containing sub-G₁ DNA content.

2.6. Isolation of total RNA and RT-PCR

Total RNA was isolated from RAW 264.7 cells using Easy-Blue (iNTRON Biotechnology, Sungnam, Republic of Korea.) according to the manufacturer's instruction. RNA extracts were reverse-transcribed by M-MLV reverse transcriptase kit (BioNEER, Daejeon, Republic of Korea). In brief, cDNA synthetic was amplified using specific primers of β -actin (forward 5'-TGTGATGGTGGGAATGGGTCAG-3' and reverse 5'-TTTGATGTCACGCACGATTTC-3') and iNOS (forward 5'-CCTCCTCC ACCCTACCAAGT-3' and reverse 5'-CACCCAAAGTGCTTCAGTCA-3'). The following PCR conditions were used: β -actin, 25 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extended at 72 °C for 30 s; iNOS, 24 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and extended at 72 °C for 30 s.

2.7. Western blot analysis

Total cellular protein extracts were prepared by PRO-PREP protein extraction kit (iNTRON Biotechnology). Cells lysate samples were centrifuged $16,000 \times g$ at 4 °C for 20 min to gain the supernatants. Cytoplasmic and nuclear protein extracts were prepared using NE-PER nuclear and cytosolic extraction reagents (Pierce, Rockford, IL). Protein samples were collected and protein concentrations detected by a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Protein samples were stored at –80 °C or immediately used in western blot analysis. Briefly, the proteins were separated on SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Amersham, Arlington Heights, IL). Proteins were monitored using an enhanced chemiluminescence detection system (Amersham).

2.8. Electrophoretic mobility shift assay (EMSA)

Transcription factor-DNA binding activity assays were carried out with nuclear protein extract. Synthetic complementary NF- κ B (5'-AGTTGAGGGGACTTCCAGGC-3', Santa Cruz Biotechnology)-binding oligonucleotides were 3'-biotinylated by the biotin 3'-end DNA labeling kit (Pierce) according to the manufacturer's instructions and annealed for 30 min at 37 °C. Samples were loaded onto native 4% polyacrylamide gels pre-electrophoresed for 60 min in 0.5X Tris borate/EDTA buffer on ice and transferred onto a positively charged nylon membrane (Hybond™-N+) in 0.5X TBE buffer at 100 V for 1 h on ice. The transferred DNA-protein complex was cross-linked to the membrane at

120 mJ/cm². Horseradish peroxidase-conjugated streptavidin was utilized according to the manufacturer's instructions to monitor the transferred DNA-protein complex.

2.9. NO assay

RAW 264.7 cells (1×10^5 cells/ml) were plated into 24-well plates and treated with the indicated concentrations of FA for 24 h. The cell culture supernatants were collected and assayed for NO generation by Griess reagent. In brief, the samples were mixed with Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride) and then incubated at room temperature for 30 min. The absorbance was measured at 540 nm on a microplate reader (Thermo Electron Corporation, Marietta, OH). Amount of NO was measured using a dilution of sodium nitrite as a standard.

2.10. Statistical analysis

The images were visualized with Chemi-Smart 2000 (VilberLourmat, Marine, Cedex, France). Images were captured using Chemi-Capt (VilberLourmat) and transported into Photoshop. All bands were shown a representative obtained in three independent experiments and quantified by Scion Imaging software (<http://www.scioncorp.com>). Statistical analyses were conducted using SigmaPlot software (version 12.0). Values were presented as mean \pm standard error (SE). Significant differences between the groups were determined using the unpaired one-way ANOVA test. Statistical significance was regarded at *, $p < 0.05$.

3. Results

3.1. FA increases proliferation of RAW 264.7 cells

In order to investigate whether FA regulates cell metabolic activity and proliferation, RAW 264.7 cells were treated with the indicated

concentrations of FA for 24 h. Treatment with highest concentration (20 μ g/ml) of FA increased metabolic activity by approximately 20% compared to that of the untreated control cells, and 10 μ g/ml and 15 μ g/ml FA also slightly increase the activity (Fig. 2A). Additionally, to evaluate whether FA-mediated increase of metabolic activity is associated with cell proliferation, total cell numbers were counted in the presence of FA. Dissimilar to MTT data, 20 μ g/ml FA only significantly increased cell proliferation and distinct change in the cell numbers was not observed at 10 μ g/ml and 15 μ g/ml FA (Fig. 2B), suggesting that FA markedly activates cell metabolic activity and proliferation. Western blot analysis showed no cleaved PARP in FA-treated cells; however, the H₂O₂-treated positive control cells showed significantly increased PARP cleavage, sign of apoptosis (Fig. 2C). For a detail evaluation of the extent of apoptosis induced by FA, we performed a DNA fragmentation assay. No fragmented DNA was detected in any FA-treated groups compared to the findings in the H₂O₂-treated positive control group (Fig. 2D). These data indicate that FA enhances the proliferation of RAW 264.7 cells without any cytotoxic effects.

3.2. FA enhances NO production without LPS contamination

To evaluate the effects of FA on NO production, RAW 264.7 cells were treated with the indicated concentrations of FA for 24 h. NO production was low in the untreated control cells; however, treatment with FA substantially increased the level of NO production in a dose-dependent manner (Fig. 3A). NO release was $8.1 \pm 1.5 \mu$ M, $12.5 \pm 1.9 \mu$ M, $16.3 \pm 2.1 \mu$ M, $20.6 \pm 3.1 \mu$ M, $20.9 \pm 1.1 \mu$ M, and $24.0 \pm 1.4 \mu$ M upon treatment with 0 μ M, 2.5 μ M, 5.0 μ M, 10.0 μ M, 15.0 μ M, and 20.0 μ M FA, respectively. In addition, RT-PCR data showed that treatment with FA significantly increased the expression of iNOS at 6 h (Fig. 3B). Consistent with the results of iNOS RT-PCR, western blot analysis also indicated that treatment with FA increased the protein expression of iNOS in a dose-dependent manner at 24 h (Fig. 3C). Next, we evaluated whether FA-induced NO production was due to LPS contamination. Stimulation with LPS significantly increased NO production ($29.5 \pm 2.3 \mu$ M);

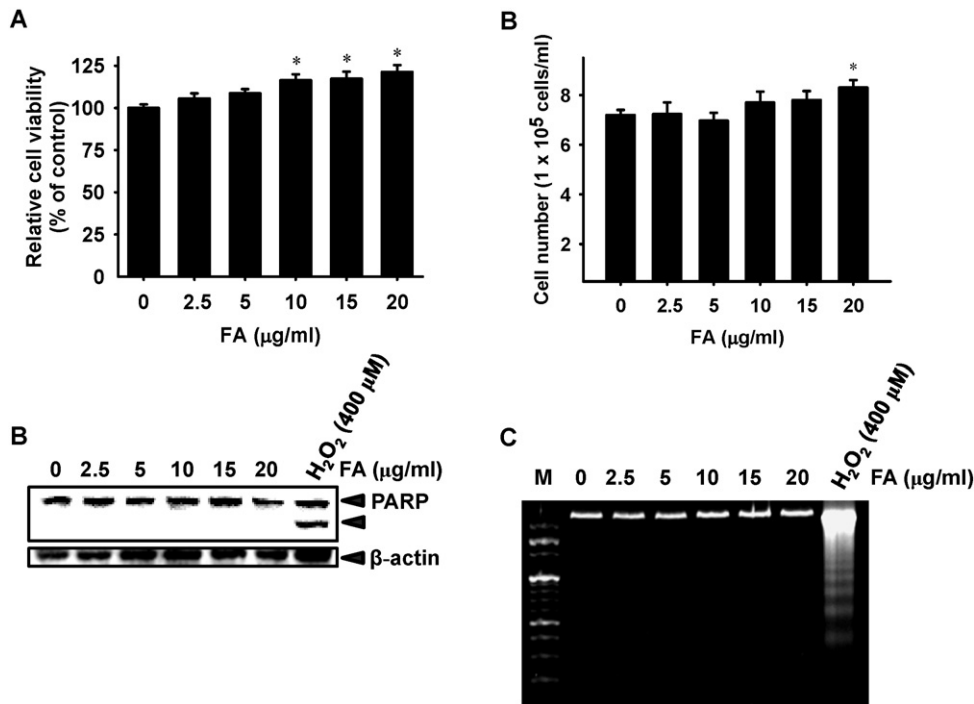


Fig. 2. FA increases the viability of RAW 264.7 cells. RAW 264.7 cells were seeded at 1×10^5 cells/ml for overnight and treated with the indicated concentrations of FA for additional 24 h. (A) Cell metabolic activity was evaluated by an MTT assay. (B) Total cell numbers were validated by counting viable cell number. (C) After the indicated treatment for 24 h, apoptotic protein PARP was detected by western blot analysis. (D) In a parallel experiment, fragmented DNAs were isolated from the stimulated RAW 264.7 cells and analyzed on 1.5% agarose gel. Statistical significance was determined by one-way ANOVA (*, $p < 0.05$ vs. untreated control).

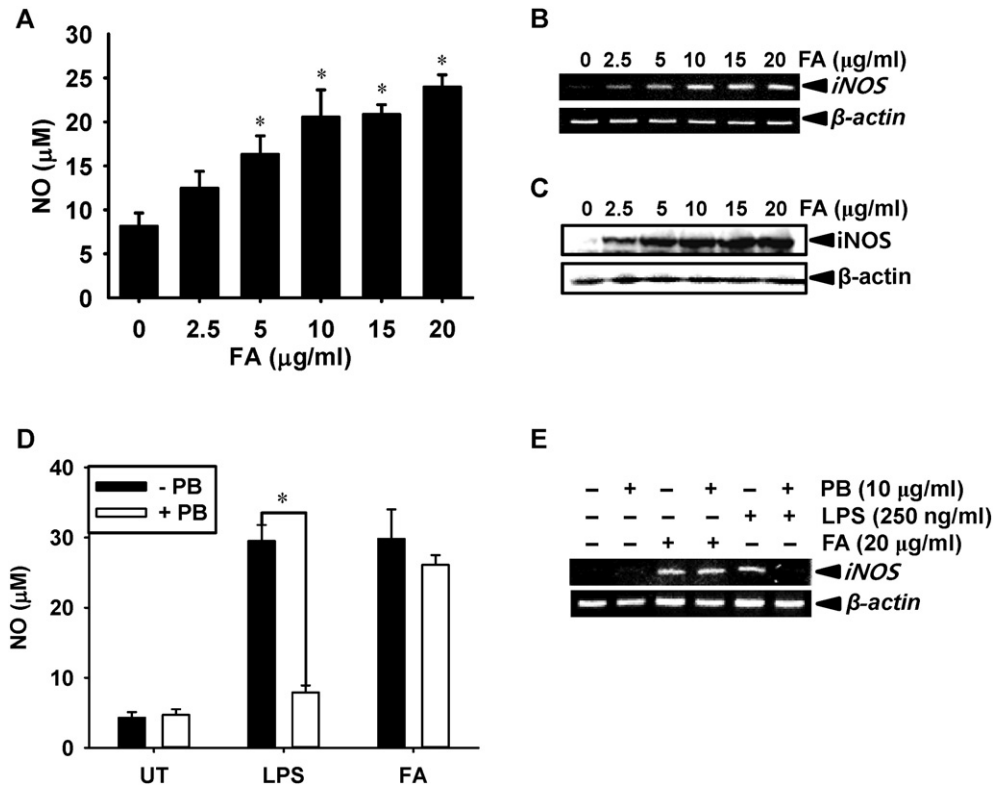


Fig. 3. FA induces NO production in accordance with iNOS expression in RAW 264.7 cells. RAW 264.7 cells were seeded at 1×10^5 cells/ml and treated with the indicated concentrations of FA. (A) The amount of NO production in the medium was measured by Griess reaction at 24 h. (B) Total cellular RNA was subjected to RT-PCR at 6 h and the amount of PCR product was determined using 1.5% agarose gel electrophoresis. (C) In a parallel experiment, cell lysates were prepared at 24 h and western blot analysis was executed using antibodies specific for iNOS. β -Actin was used as an internal control for the western blot analysis and RT-PCR. In the presence of polymyxin B (PB), FA-induced NO production (D) and iNOS mRNA expression (E) were analyzed. Statistical significance was determined by one-way ANOVA (*, $p < 0.05$ vs. untreated control (A) or free-PB condition (D)).

however, pretreatment with an LPS inhibitor, PB, markedly decreased NO production ($7.9 \pm 1.0 \mu\text{M}$, Fig. 3D). FA also enhanced NO production ($29.8 \pm 4.2 \mu\text{M}$) to a level equivalent to that observed with LPS; however, pretreatment with PB had no significant influence on FA-induced NO

production ($26.1 \pm 1.4 \mu\text{M}$). Similarly, the PB pretreatment did not downregulate FA-induced iNOS expression at 6 h (Fig. 3E), suggesting that FA increases iNOS expression and NO production, not through LPS contamination.

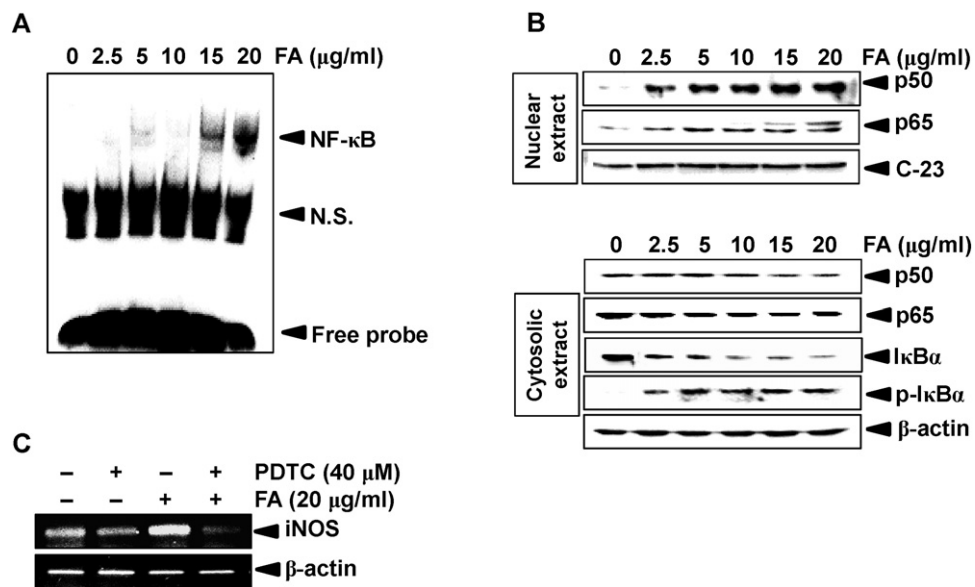


Fig. 4. FA stimulates NF- κ B activity to enhance iNOS mRNA expression. RAW 264.7 cells were seeded at 1×10^5 cells/ml. (A) The cells were treated with FA for 30 min and then the nuclear extracts were assayed for specific DNA-binding activity of NF- κ B. (B) The levels of p50, p65, I κ B α , and p-I κ B α in the nuclear (top) and cytosolic (bottom) compartments were assessed by western blot analysis at 30 min. (C) RAW 264.7 cells (2×10^5 cells/ml) were incubated with 40 μM PDTC for 2 h before treatment with 20 $\mu\text{g/ml}$ FA. The total RNA was isolated at 6 h and RT-PCR analyses of iNOS mRNA expression were performed. C-23 and β -actin were used as the internal controls of the nucleus and the cytosol.

3.3. FA increases expression of iNOS mRNA by inducing NF- κ B activation

To investigate whether FA regulates NF- κ B activation in RAW 264.7 cells, we performed an EMSA and western blot analysis. The EMSA showed that FA significantly upregulated the DNA-binding activity of NF- κ B in a concentration-dependent manner at 30 min (Fig. 4A). In a parallel experiment, the expression levels of p65 and p50 were determined in both the cytoplasmic and the nuclear fractions. FA treatment significantly decreased the expression of p65 and p50 in the cytosolic compartment (top) and subsequently upregulated their translocation into the nucleus (bottom, Fig. 4B). FA also induced the degradation and phosphorylation of I κ B α in the cytosolic compartment (bottom), suggesting that FA induces the nuclear localization of subunits p65 and p50 by enhancing phosphorylation-mediated degradation of I κ B α . In addition, our data indirectly demonstrated that a specific NF- κ B inhibitor PDTC inhibited FA-induced iNOS expression (Fig. 4C). These data indicate that FA enhances iNOS expression by inducing NF- κ B activation.

3.4. FA-CM triggers apoptotic death of MCA-102 fibrosarcoma cells

In order to investigate whether FA-CM from RAW 264.7 cells has cytotoxic effect, MCA-102 fibrosarcoma cells were treated with the indicated percentages of FA-CM for 24 h. FA-CM significantly reduced MCA-102 fibrosarcoma cell viability in a dose-dependent manner (Fig. 5A). According to the MTT assay, treatment with 100% FA-CM

reduced viability of MCA-102 fibrosarcoma cells to $56 \pm 6\%$. Under the light microscope, MCA-102 fibrosarcoma cells also exhibited apoptotic characteristics and low cell density after treatment with FA-CM (Fig. 5B). Furthermore, to evaluate the level of apoptosis induced by FA-CM, we performed DNA fragmentation assay. FA-CM gradually increased DNA fragmentation in a concentration-dependent manner (Fig. 5C). In addition, analysis of cell cycle distribution showed that treatment with FA-CM increased the sub-G₁ DNA content (Fig. 5D). Western blot analysis also verified that FA-CM significantly induced loss of procaspase-3 and cleavage of PARP (Fig. 5E). These data indicate that FA-CM inhibits proliferation of and induces cell death in MCA-102 fibrosarcoma cells.

3.5. FA-induced NO is not the key player in MCA-102 fibrosarcoma cell apoptosis

In order to evaluate the cytotoxic effect of FA-induced NO production, we measured FA-induced NO release from RAW 264.7 cells and FA-CM-mediated apoptotic effects in MCA-102 fibrosarcoma cells in the presence of an NO inhibitor L-NMMA. As presumed, L-NMMA significantly inhibited FA-induced NO release in RAW 264.7 cells from $16.8 \pm 2.1 \mu\text{M}$ to $8.2 \pm 1.2 \mu\text{M}$ (Fig. 6A); however, contradictory to our hypothesis, L-NMMA slightly restored (approximately 10%) FA-CM-mediated MCA-102 fibrosarcoma cell apoptosis (Fig. 6B). These data indicate that FA-induced immune-regulating mediators, in addition to NO, are involved in FA-CM-mediated apoptosis effects.

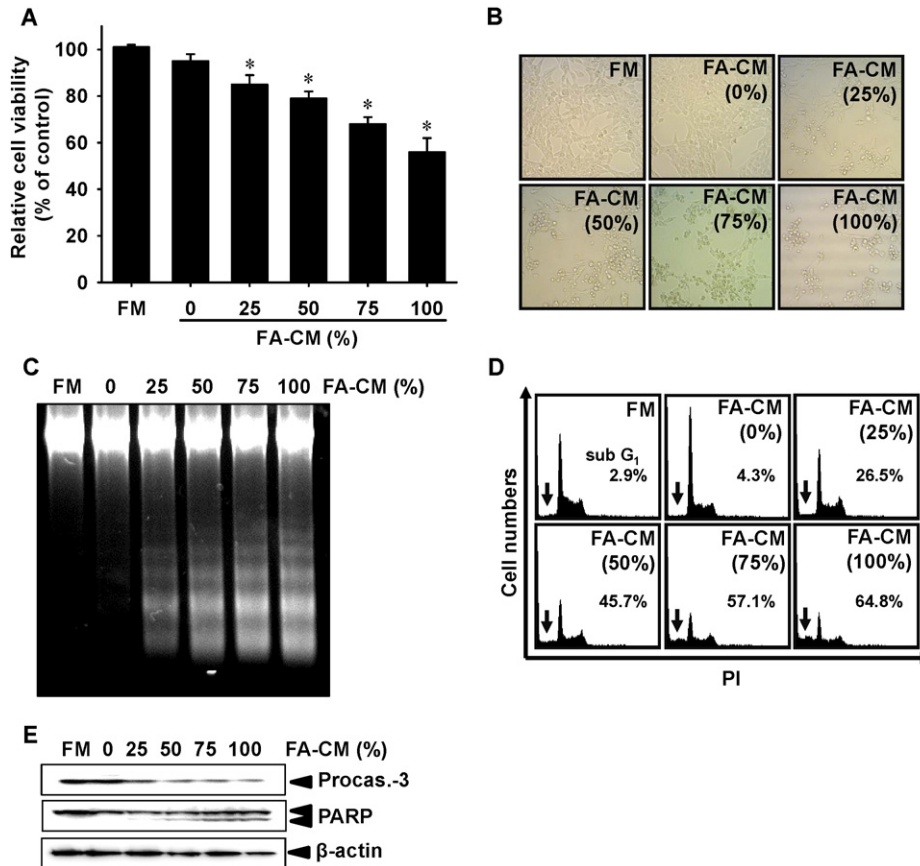


Fig. 5. FA-stimulated RAW 264.7 cell culture medium (FA-CM) induces apoptosis of MCA-102 fibrosarcoma cells. RAW 264.7 cells were seeded at 1×10^5 cells/ml and treated with the indicated concentrations of FA. The culture media from RAW 264.7 cells (FA-CM) were collected and diluted with fresh media (FM). MCA-102-culturing media were changed by the indicated percentage of FA-CM for 24 h. FM were used as a control. (A) MCA-102 cell viability was evaluated by an MTT assay. (B) The cellular morphology of MCA-102 fibrosarcoma cells was examined under a light microscope after treatment with FA-CM for 24 h. (C) Fragmented DNAs were isolated at 24 h and analyzed on 1.5% agarose gel. (D) Sub-G₁ populations were detected by flow cytometry. (E) In a parallel experiment, cell lysates were prepared and western blot analysis was executed using antibodies specific for indicated proteins. Statistical significance was determined by one-way ANOVA (*, $p < 0.05$ vs. untreated control).

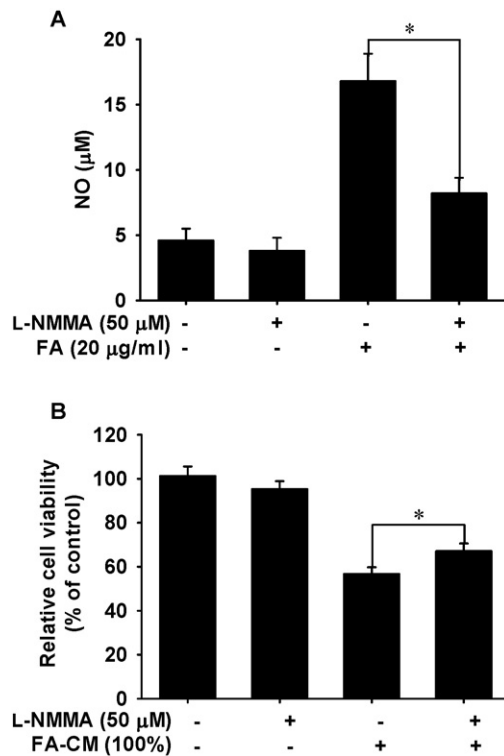


Fig. 6. FA-CM-stimulated MCA-102 cell death is not dependent on NO production. (A) RAW 264.7 cells were seeded at 1×10^5 cells/ml and treated with 50 µM L-NMMA before incubation with 20 µg/ml FA. The amount of NO production in the medium was measured by Griess reaction at 24 h. (B) MCA-102 fibrosarcoma cells were treated with FA-CM in the presence or absence of L-NMMA. MCA-102 cell viability was evaluated by an MTT assay at 24 h. Statistical significance was determined by one-way ANOVA (*, $p < 0.05$ vs. FA-treated group (A) and FA-CM-treated group).

3.6. FA-CM enhances death in various human cancer cells

In order to evaluate whether FA-CM possesses anti-proliferative effect in broad spectrum of cancer cells, we treated 100% FA-CM in various human cancer cells such as Hep3B, LNCaP, and HL60 cells. After treatment with 100% FA-CM for 24 h, we found that relative cell viability significantly decreased in LNCaP cells and HL60 cells to approximately 70% (Fig. 7A, middle and bottom panel). Hep3B also statistically downregulated in cell viability in response to FA-CM (Fig. 7A, top), but anti-cell viability of FA-CM in Hep3B was weaker than that of LNCaP and HL60 cells. Additionally, we determined the change of cell morphology under microscope in response to FA-CM. Shrinkage of LNCaP cells and HL60 cells was seen in the presence of FA-CM, but direct treatment of FA showed the intact cells compared to the untreated control (Fig. 7B). Hep3B cells sustained intact shape in response to FA-CM. These data indicate that FA-CM inhibits cancer cell proliferation, but different effect according to cancer cell types.

4. Discussion

NO is a multifunctional regulator of biological processes such as vasodilation, secondary signal transmission, and macrophage-mediated immune response. In particular, high levels of NO have been known to trigger cancer cell apoptosis by modulating caspase activity, upregulating p53, and inducing mitochondrial dysfunction [13,14]. In the current study, we first demonstrated that FA induced NO production in macrophage cells by regulating NF-κB activity and FA-CM triggered MCA-102 fibrosarcoma cell apoptosis; however, FA did not directly induced MCA-102 cell death. FA-CM-induced MCA-102 cell death was slightly restored by the NO inhibitor L-NMMA, although L-NMMA significantly

inhibited NO production in macrophage cells. Therefore, other apoptosis-inducing factors may be involved in FA-CM-mediated cell death. Nevertheless, because we, in this study, used RAW 264.7 cells that are immortalized leukemic cells possessing macrophage function, we cannot exclude the possibility that RAW 264.7 cell-specific substances induce MCA-102 cell death. Moreover, we found that FA-CM downregulated various human cancer cells such as Hep3B cells, LNCaP cells, and HL60 cells *in vitro*. Therefore, we need to verify whether FA-stimulated real macrophages kill cancer cells *in vivo* and what substances are major for cancer cell death.

The transcription factor NF-κB plays a critical role in the onset of immune-regulating responses by modulating the expression of pro-inflammatory genes such as iNOS [15]. We demonstrated that FA-induced iNOS in macrophage cells is intricately regulated by activating NF-κB. Nevertheless, we didn't understand whether FA directly regulates NF-κB activity because central regulators of NF-κB are known as IκB kinases and NF-κB essential modulator [16–18]. We should also find what receptor is a target for FA-induced immunostimulation in macrophages. In contrast to our data, Chien et al., recently reported that FA inhibited homocysteine-induced cyclooxygenase-2 expression by suppressing NF-κB activity, which consequently downregulated prostaglandin E₂ production [9]. The discrepancy is because of the level of total carbohydrates because Schepetkin et al., showed that each fraction of FA possessed different mode in ROS generation according to amount of carbohydrate contents [11]. In addition to NF-κB, various other transcription factors, including activation protein-1 [19], mitogen activated protein kinase [20], and cyclic AMP-response element-binding protein [21] may regulate the expression of immune-regulating genes. Therefore, further studies are needed to evaluate the relationships among the various signaling pathways and transcription factors involved in the FA-induced expression of iNOS and other immune-regulating genes.

Under stress conditions, NO production influences the expression of gene that regulates immune responses in host or pathogens and induces cell death [22]. Snyder et al. reported that NO-induced cell death involves the intrinsic apoptotic pathway and induces the release of cytochrome c from the mitochondria [23]. Additionally, Oyadomari et al. demonstrated that NO induces endoplasmic reticulum (ER) stress by depleting Ca²⁺ flow and leads to apoptosis [24]. In this study, FA substantially increased NO production and FA-CM containing high levels of NO enhanced MCA-102 fibrosarcoma cell apoptosis; however, apoptotic cell death was slightly restored by the NO inhibitor L-NMMA. According to Rapozzi's study [25], dual effect of NO regulates cytoprotection and cytotoxicity; low levels of NO enhance cell proliferation and resistance to cytotoxic drugs by activating NF-κB/Snail/YY1. In contrast, high levels of NO induce apoptosis and sensitization to cytotoxic drugs. Therefore, we cannot rule out the possibility that FA-CM-induced NO production is not sufficient to kill MCA-102 fibrosarcoma cells. Another hypothesis is that other immune-regulating mediators may be involved in the anti-cancer activity of FA because other well-known candidates such as reactive oxygen species (ROS), TNF-α, and IL-1β are simulated by LPS, which stimulate anti-cancer activity [26]. In peculiar, Soler et al. reported that TNF-α triggers apoptotic events and the programmed cell death is inhibited in TNF-α-receptor knockout mice [27]. Additionally, previous studies have shown that LPS-induced ROS and some interleukins (ILs) such as IL-2, IL-4, and IL-18 also cause cancer cell apoptosis [28–30]. Jennifer et al. showed that IL-4 increases apoptotic cell death in breast cancer cells [31]. Evidence has also indicated that IL-18 exerts anti-cancer effects by inhibiting tumor growth and angiogenesis [32]. Moreover, Vaillier et al. showed that the presence of IL-2 and LPS or both in the culture supernatant enhances cytotoxicity against YAC-1 lymphoma cell lines [33]. These data indicate that FA-CM possesses considerable apoptosis-inducing regulators to kill MCA-102 fibrosarcoma cells. Additionally, Schepetkin et al. reported that FA increases ROS generation, which consequently stimulates the apoptotic signal pathway [11]. While FA increases ROS generation in the previous

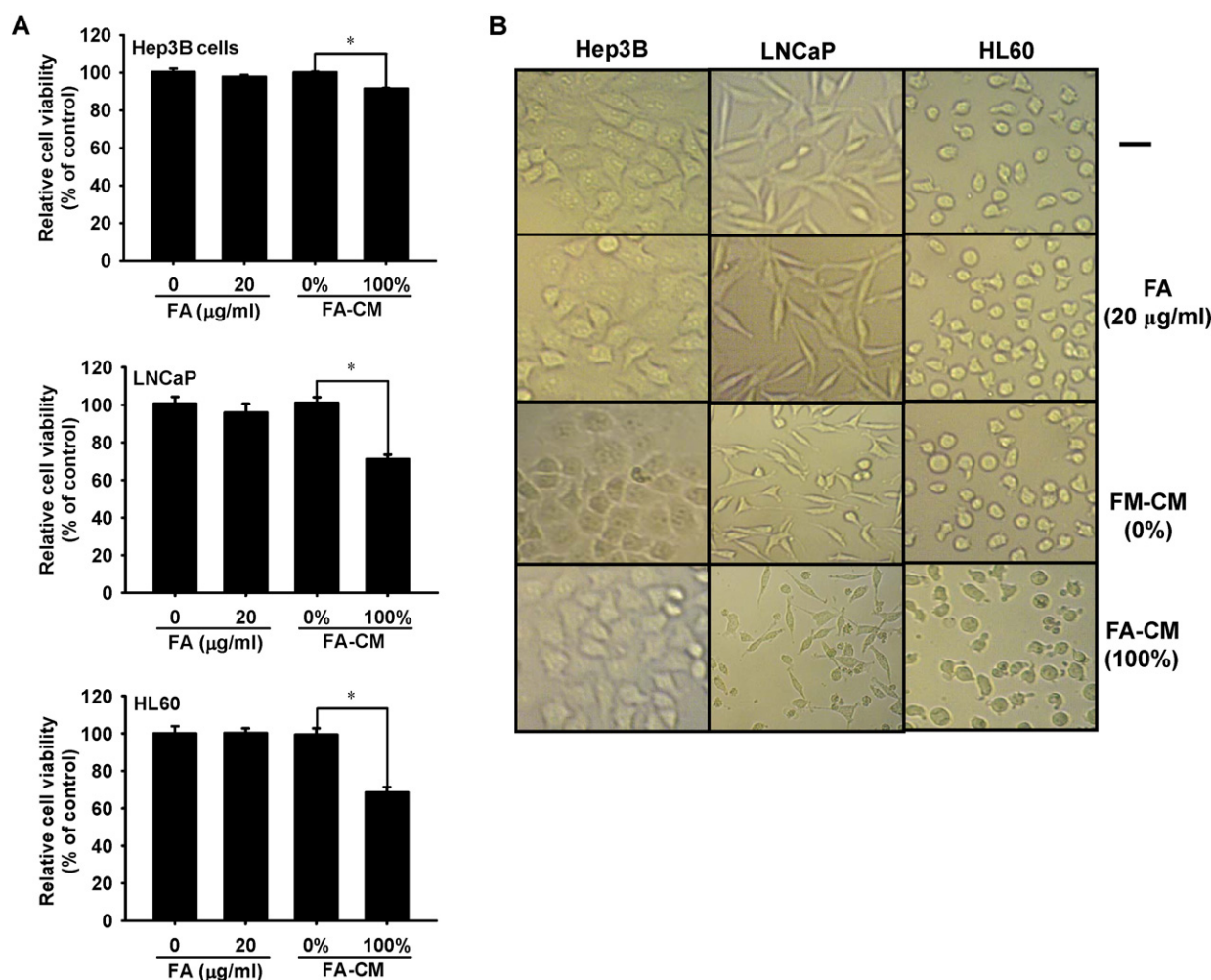


Fig. 7. FA-CM induces death in various human cancer cells. Hep3B cells, LNCaP cells, and HL60 cells were seeded at 1×10^5 cells/ml and treated with FA only and 100% FA-CM for 24 h. (A) Cell metabolic activity was evaluated by an MTT assay. Statistical significance was determined by two-way ANOVA (*, $p < 0.05$ vs. 0% FA-CM-treated group). (B) In a parallel experiment, the cellular morphology of Hep3B cells, LNCaP cells, and HL60 cells was examined under a light microscope after treatment with FA only and 100% FA-CM for 24 h.

study [11], we did not measure FA-induced ROS generation and the levels of ILs in macrophage cells. Therefore, further studies are warranted to investigate the direct effect of FA on the release of ROS and ILs.

In summary, our data indicated that FA significantly induces NO production by stimulating the NF- κ B pathway without any cytotoxicity. While we did not determine the ultimate role of FA-induced NO production in apoptotic death of MCA-102 fibrosarcoma cells, the apoptosis-inducing potential of FA-CM indicates the possibility of using it as a therapeutic agent for cancer therapy. Nevertheless, we need further study on chemical property of FA and whether FA stimulates immune system *in vivo*.

Author disclosure statement

The authors declare to have no conflict of interest at all.

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