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Arachidonic Acid Induces Apoptosis Under Serum-Free Conditions by Blocking PAK1-Mediated PUMA Suppression

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Abstract

Arachidonic acid is an essential lipid, which should be supplied by food uptake or biosynthetic pathway. In addition, it can be converted into Prostaglandin E2 (PGE2) by COX-2, which is well known to be related with inflammation as well as cancer development. In fact, treatment of PGE2 can induce colon adenoma and COX-2 expression is elevated in colon cancers. However, the relevance between arachidonic acid itself and cancer has not been investigated. Here we provided evidences that arachidonic acid can induce cell death *via* inhibition of PAK1-PUMA binding. Since serum starvation induces PUMA-mediated apoptosis, arachidonic acid-induced cell death is obviously detected in Serum-Free media condition (SF). Moreover, arachidonic acid can induce SF-induced apoptosis in Smad4-deficient cells, suggesting that arachidonic acid can work in Smad4-downstream pathway. Indeed, we can observe the selective suppression of PAK1-PUMA binding by AA treatment through GST-pull down assay. In addition, BSA can block the arachidonic acid-induced cell death like as Smad4-mediated apoptosis, whereas COX-2 inhibitor such as celecoxib shows a synergic effect in cancer cell death. Our results indicate that arachidonic acid is an important apoptosis trigger in human cancer cells. Together, these findings suggest that arachidonic acid could be a useful plausible target for cancer treatment.

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Introduction

Arachidonic Acid (AA) is an essential lipid that can be used as plasma membrane component and precursor of various kinds of lipid metabolites [1-3]. One of well-known metabolite of AA is Prostaglandin E2 (PGE2), which is closely related with inflammation. PGE2 can induce diverse inflammation reaction and is known to be related with inflammation-related human cancers including colon, pancreatic and breast cancers [4]. Indeed, Cyclooxygenase-2 (COX-2), AA converting enzyme into PGE2, shows elevated expression in several kinds of human cancers [5-11]. Thus, clinical trials using COX-2 inhibitors (non- steroid anti-inflammation drugs such as aspirin or celecoxib) has been carried for prevention or curing the colon cancer [12-18]. According to recent data, long term treatment of aspirin can prevent colon cancer [12-15]. In addition, celecoxib, which has been expelled from market because of undesired effect such as heart attack, shows the anti-cancer effect on inflammation-related cancers [19]. However, effect of AA on human cancer has not been investigated until now. In addition, it has not clearly demonstrated how COX-2 inhibitor can induce cancer cell death.

Thus, we checked the effect of AA on human cancer cells and found that AA could induce apoptosis through the inhibition of PAK1-PUMA binding. In recent, we revealed that serum starvation-induced cell death is achieved by induction of Smad4 [20]. Induced Smad4 in response to SF suppresses the PAK1 activity, which can block the PUMA-induced cell death. Thus, in human cancer, Smad4 is frequently deleted.

Since AA-induced cell death is occurred under serum free condition, we postulate that working pathway would be related with Smad4-PAK1-PUMA axis. Here we provide the evidences about role of AA in apoptosis pathway of Smad4-PAK1-PUMA.

Materials and Methods

Cell lines and reagents

All of the human cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and the Korean Cell Line Bank (KCLB, Seoul, South Korea). Cells were maintained in RPMI-1640 or DMEM supplemented with 10% FBS and 1% penicillin and were grown at 37°C in chambers containing 5% CO_2. HCT116 (p53 ±) cells and its isogenic cell lines (PUMA-/- and BAX-/-) were provided by Dr. B. Vogelstein [21-24]. Cyclohexamide, Arachidonic acid, Sulindac and PGE2 were purchased from Sigma (St. Louis, MO, USA). AG879 was obtained from Calbiochem (Merck, Darmstadt, Germany), and Okadaic acid was purchased from Enzo Life Science (Farmingdale, NY, USA).

Western blot analysis, IP and GST pull-down assays

Proteins were extracted from cells using Radio Immuno Precipitation Assay (RIPA) buffer and were separated *via* SDS-PAGE. Proteins were transferred to PVDF membranes, and routine western blot protocols were followed. Blotted membranes were incubated with antibodies against Smad4 (B-8; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PUMA (PC686; Calbiochem), PAK1 (2602; Cell Signaling Technology, Danvers, MA, USA), Actin (I-19; Santa Cruz Biotechnology) and GST (B-14; Santa Cruz Biotechnology). HRP-conjugated goat anti-mouse, goat anti-rabbit and mouse anti-goat antibodies (Pierce, Thermo Fisher Scientific, Inc., Rockford, IL, USA)

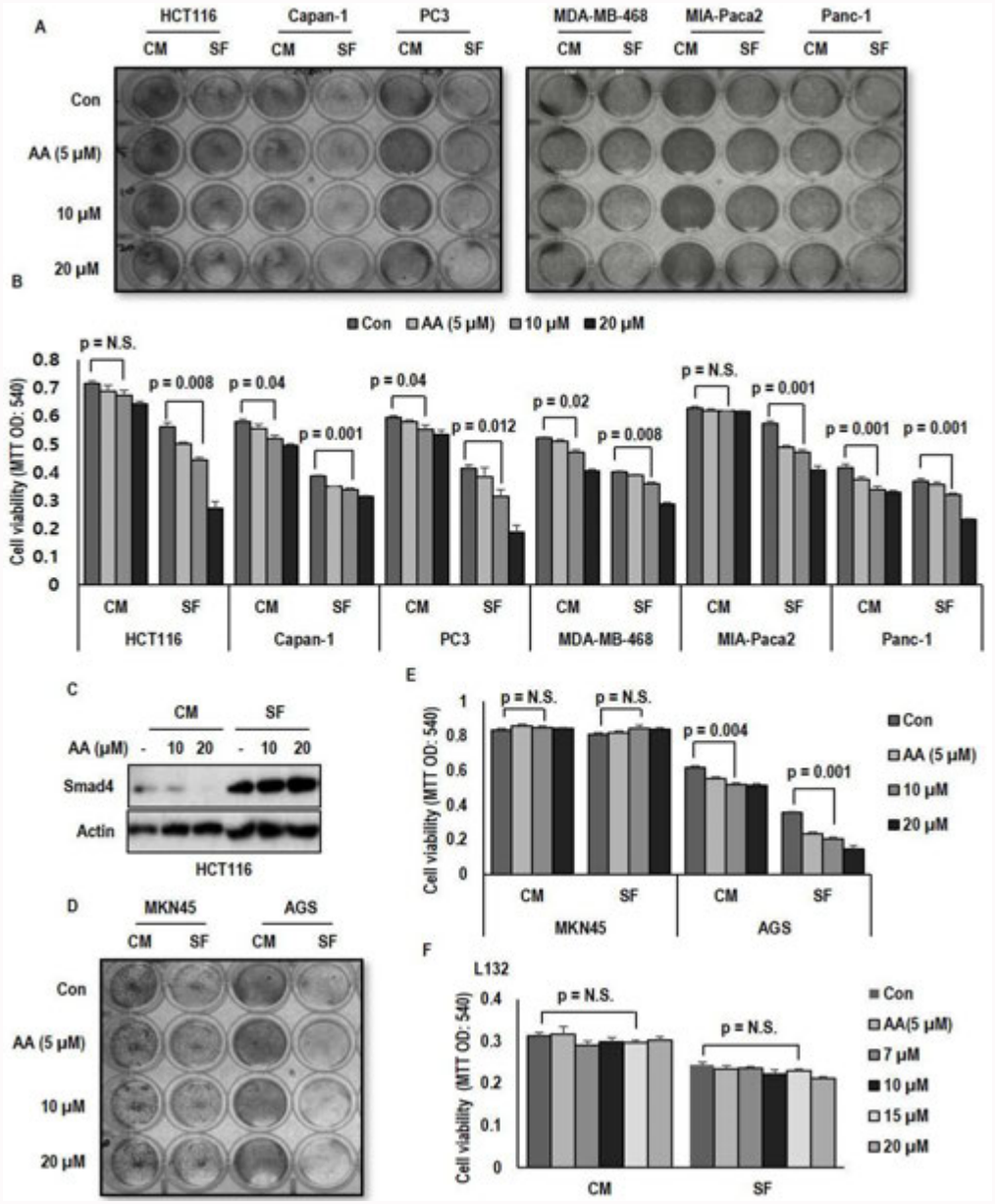

Figure 1: Arachidonic acid decreases cell viability under conditions of starvation. (A and B) AA induces cell death under SF conditions, and HCT116 and PC3 cells exhibited the highest sensitivity. Following treatment with the indicated concentration of AA for 48 h, Trypan Blue (TB) staining (A) and MTT assays (B) were performed. CM: Complete Media; SF: Serum-Free Media. (C) Smad4 expression is not altered by AA treatment. HCT116 cells were treated with 10 μM and 20 μM AA for 6 h, and western blot analyses were conducted with the indicated antibodies. Actin was used as the loading control. (D and E) MKN45 cells were resistant to AA under SF conditions. Two gastric cancer cell lines were treated with various doses of AA for 48 h, and TB staining (D) and MTT assays (E) were performed to measure cell viability. (F) AA had no effect on the viability of non-cancerous L132 cells. The MTT assay was performed following 48 h of AA treatment.

were used as secondary antibodies.

For the IP analysis, whole cell lysates were incubated with proper antibodies for 2 h at 4°C and then with protein A/G agarose beads (Invitrogen, Carlsbad, CA, USA) for 2 h. After centrifugation and washing with Phosphate-Buffered Saline (PBS), the immuno complexes were separated by SDS-PAGE and analyzed *via* WB. The GST pull-down assay was conducted using agarose bead-conjugated GST-Smad4 (MH1) and PUMA (full-length). After incubating proteins for 2 h at 4°C, the precipitated proteins were also separated by SDS-PAGE and analyzed *via* WB.

Cell viability measurements

Trypan Blue (TB) staining was performed to measure cell viability. After treatment with the indicated chemicals for 48 h, cells were fixed with 1% paraformaldehyde for 20 min. Cells were then washed twice with PBS and stained with 0.05% Trypan blue solution (Gibco, BRL, Paisley, UK). For the MTT assay, cells were incubated with 0.5 mg/ml of MTT solution (Calbiochem) at 37°C for 4 h. After the excess solution was removed, cells were dissolved in DMSO (200 µl) and quantified by measuring the absorbance at 540 nm.

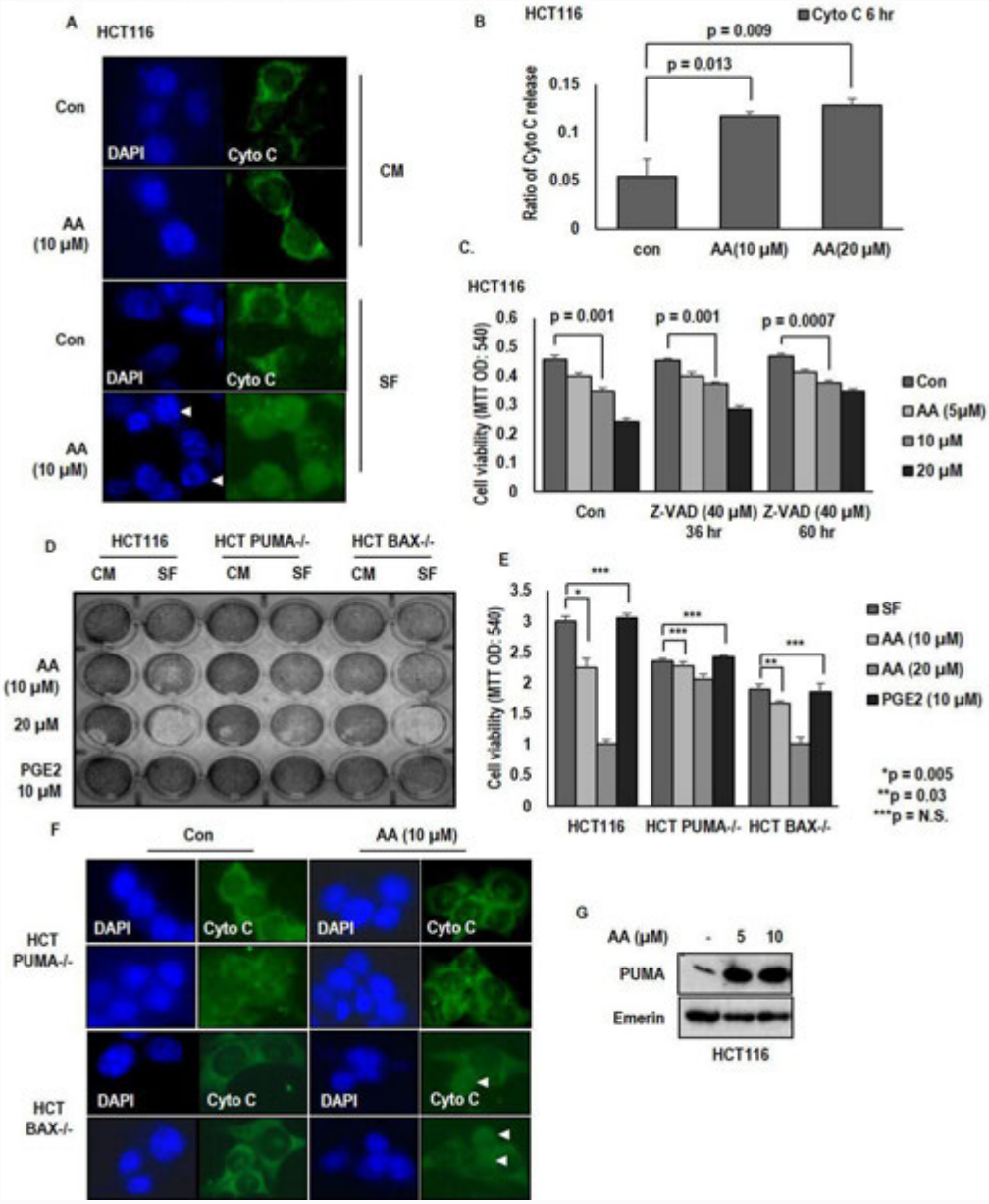

Figure 2: Arachidonic acid induces apoptosis. (A) AA treatment under serum-free conditions induces cytochrome C release and DNA condensation (arrowheads). HCT116 cells were treated with AA (10 µM) for 6 h. After methanol fixation, cells were stained with anti-cytochrome C (Green) and DAPI (Blue). (B) Following staining, released cytochrome C was counted and analyzed. (C) The pan-caspase inhibitor (Z-VAD) blocks cell death which was stimulated by AA. HCT116 cells were treated with Z-VAD (40 µM) and indicated concentrations of AA. After 48 h, cell viability was measured by MTT assay. (D and E) In contrast to HCT116 and HCT BAX-/- cells, HCT PUMA-/- cells are resistant to AA-induced cell death. Cells were incubated with the indicated concentrations of AA or PGE2 under both CM and SF conditions. After treatment for 6 h, TB staining (D) and MTT assays (E) were conducted. (F) PUMA-deficient cells (HCT PUMA-/-) did not release cytochrome c (arrowheads) following AA treatment, suggesting that apoptosis is dependent on PUMA expression but independent of BAX. Cells were incubated with AA (10 µM) for 6 h under SF conditions. Cells were then fixed with methanol and stained with anti-cytochrome C (Green) and DAPI (Blue). (G) AA induces PUMA expression. HCT116 cells were treated with the indicated concentration of AA for 6 h, and PUMA levels were analyzed by western blot assays. Emerin was used as the loading control.

Immunofluorescence staining

Prior to staining, cells on coverslips were washed and fixed with 100% methanol for 20 min at -20°C. Cells were treated with blocking solution (PBS containing anti-human Ab at a dilution of 1:500; Pierce) for 1 h and then incubated with anti-cytochrome C antibody (556432; 1:200; BD Pharmingen, San Diego, CA, USA) overnight at 4°C. Finally, the cells were incubated with FITC-conjugated secondary antibodies (1:500) for 2 h. Following DAPI staining and washing, the coverslips were mounted with mounting solution (H-5501; Vector Laboratories, Burlingame, CA, USA), and images were acquired by fluorescence microscopy (Zeiss).

Statistical analysis

The student's *t*-test was used for comparisons of two groups. *P*-value less than 0.05 was considered significant. Error bars indicate Standard Deviation (SD). Data for all figures are expressed as means ± SD of at least three independent experiments.

Results

Arachidonic acid suppresses cell viability under serum starvation conditions

To investigate the effect of Arachidonic Acid (AA) on human cancer cell survival and proliferation, we conducted Trypan Blue (TB) staining in 6 human cancer cell lines (HCT116, colon cancer; Capan-1, MIA-Paca2, and Panc-1, pancreatic cancer; PC3, prostate cancer; and MDA-MB-468, breast cancer). Although the anti-proliferative effect of AA treatment was detected in all of the cancer cell lines under SF conditions (Figure 1A), HCT116 and PC3 cells exhibited the most sensitive response to AA treatment (Figure 1A). To confirm this result, we performed the MTT assay and obtained a similar result (Figure 1B). In contrast, Capan-1 and MIA-Paca2 cells were resistant to AA-mediated anti-proliferative effects and cell death (Figure 1A, 1B). Interestingly, these cell lines have been reported to be resistant to SF-induced apoptosis [20]. Previously, we reported that SF-induced cell death is achieved *via* the Smad4-PAK1-PUMA axis and that these cell lines possess genetic defects in this pathway [20]. Therefore, we investigated the effect of AA on Smad4 expression. However, we did not observe AA-mediated Smad4 induction (Figure 1C). In a previous study, we also observed that the MKN45 gastric cancer cell line was resistant to SF-induced cell death [20], we monitored the effect of AA on two additional gastric cancer cell lines (AGS and MKN45). Unlike the AGS cell line, MKN45 cells were also resistant to AA-induced cell death (Figure 1D, 1E). This result suggests that AA may potentiate the SF-induced cell death pathway independent of the tissue type. Next, we evaluated the effect of AA on a non-cancer cell line (L132) and found that AA did not reduce its cell viability (Figure 1F). This result indicates that AA-induced cell death is not a result of random cytotoxicity.

Arachidonic acid induces apoptosis

To further understand the mechanism responsible for the AA-induced effects in cancer cells, we examined cytochrome C release, which is an important marker for apoptosis [25,26]. AA treatment of cells grown in SF media induced cytochrome C release (Figure 2A, 2B). We also observed DNA condensation *via* DAPI staining in these AA-treated cells (Figure 2A; arrowheads). Inhibition of caspase activity by the pan-caspase inhibitor (Z-VAD; [27]) led to a reduction in cell death (Figure 2C), which further indicates that the AA-mediated reduction in cell viability is a result of apoptosis. Because AA-induced cell death is increased under SF conditions (Figure 1A, 1B) and because starvation-induced cell death is a result of the Smad4-PUMA pathway [20]. We tested the involvement of PUMA in AA-induced apoptosis by employing PUMA-/- and BAX-/- HCT116 cell lines [21-23]. AA induced cell death in HCT116 cells and its isogenic BAX-/- cells. Conversely, PUMA-/- cells were completely resistant to AA-induced cell death (Figure 2D). We obtained the same result from the MTT assay (Figure 2E) and from cytochrome C staining (Figure 2F; arrowheads). Because AA can be converted into Prostaglandin E2 (PGE2) by COX-2 [28], we examined the effect of PGE2 on cell death. PGE2 did not alter the viability of any of the tested cell lines (Figure 2D, 2E), which indicated that AA, but not its metabolite, is responsible for cell death. To further investigate the role of PUMA in AA-induced cell death, we measured PUMA expression levels and found that AA could increase PUMA expression in HCT116 cells (Figure 2G). Our results strongly suggest that AA-induced cell death is mediated *via* apoptosis, which is regulated by a PUMA-mediated pathway.

Arachidonic acid suppresses PAK1 expression and activity

In a previous study, we showed that PAK1 inhibits PUMA-mediated apoptosis and that Smad4 promotes cell death by inhibiting PAK1 activity [20]; therefore, we speculated that AA-mediated apoptosis is linked to this pathway. Thus, we investigated the effect of AA on the Smad4-PAK1-PUMA signaling axis. Although AA did not induce Smad4 expression (Figure 3A), a reduction in PAK1 levels was detected in AA-treated cells (Figure 3A). Moreover, PUMA expression was apparently induced by AA (Figure 3A). We then evaluated the effect of AA on a Smad4-deficient cell line (Capan-1) and found that AA could induce PUMA and suppress PAK1 (Figure 3B). To address whether AA-induced cell death is a result of PAK1 suppression, we measured the cell viability of AG879 (PAK1 inhibitor) treated cells. The results from this experiment indicate that AA did not act synergistically with the PAK1 inhibitor (Figure 3C). This result also supported our hypothesis that the AA-induced cell death was dependent on PAK1 suppression. To determine whether AA can inhibit the kinase activity of PAK1, we determined the phosphorylation status of BAD, a confirmed PAK1 substrate [29]. As shown in Figure 3D, levels of phosphorylated BAD (p-BAD) were diminished following AA treatment, and this reduction in pBAD was independent of PGE2 expression (Figure 3D).

Arachidonic acid reduces the expression of PAK1 under serum-free conditions

The previous results demonstrated that AA induces cell death under SF conditions (Figure 1A, 1B). In contrast, we did not observe the induction of PUMA under SF conditions (Figure 3A). To understand these conflicting results, we speculated that AA could promote PAK1 inactivation due to rapid uptake of AA under SF conditions. To address this possibility, we measured the effect of AA on the protein half-life of PAK1. AA did not alter the levels of the PAK1 transcript (data not shown). As shown in Figure 3E, AA could reduce the PAK1 protein half-life from 4-6 hour to 2-4 hour in cells grown in media containing serum (complete media; CM) (Figure 3E). However, under SF conditions, AA dramatically reduced the PAK1 protein half-life to less than 2 h (Figure 3F). We obtained similar results in PC3 cells, where AA could substantially reduce the PAK1 expression level and protein half-life (Figure 3G). Because our hypothesis states that serum factor(s) may interfere with AA uptake, serum-free conditions should also increase the sensitivity of cells to AA-mediated PAK1 reduction. To test this hypothesis, we treated

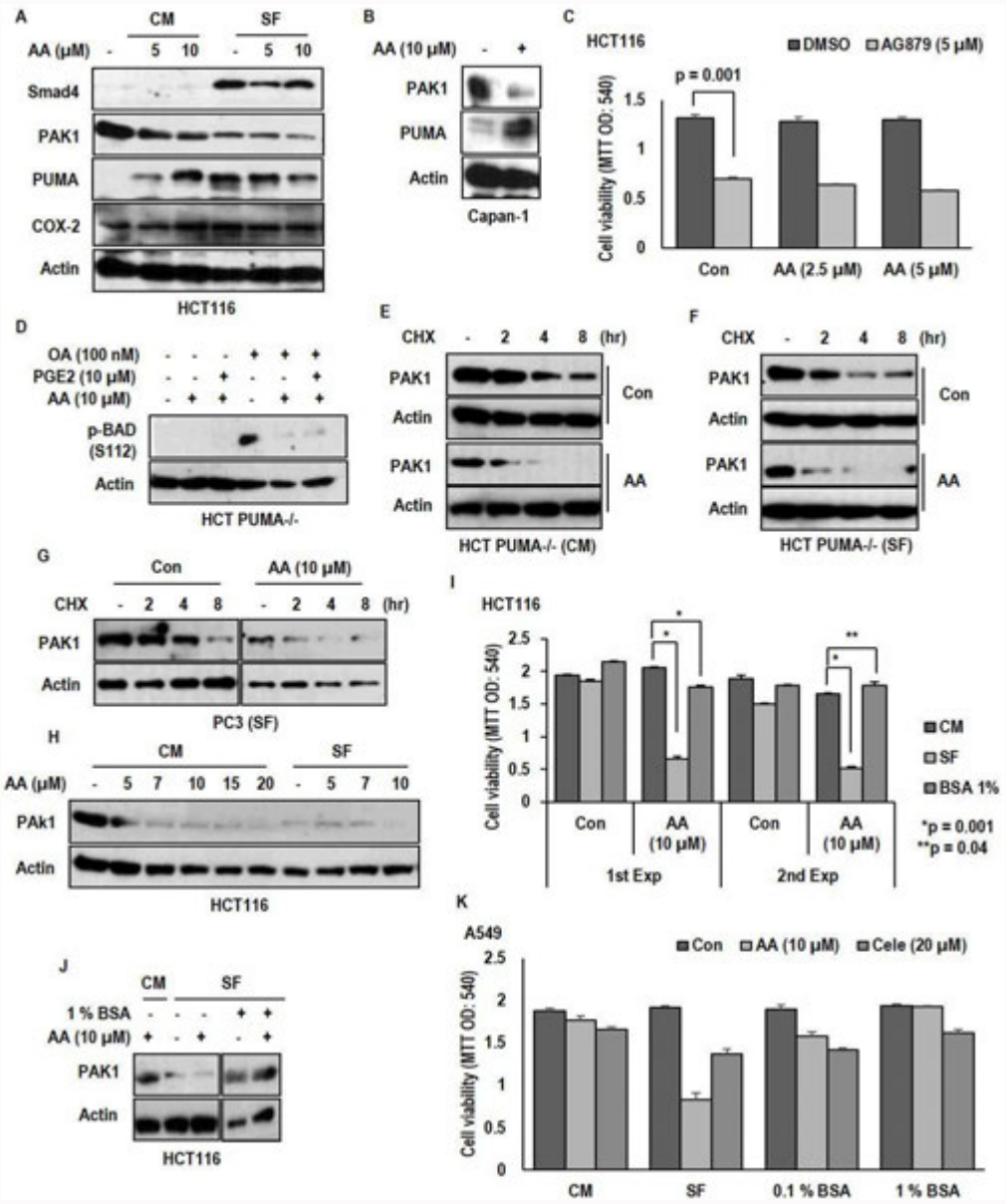

Figure 3: Arachidonic acid suppresses PAK1 expression and activity. (A) AA reduced the expression of PAK1 in a dose-dependent manner, but not Smad4. AA induced the expression of PUMA under CM conditions. HCT116 cells were treated with AA and western blot analyses were conducted using the indicated antibodies. Actin was used as the loading control. (B) Smad4-deficient cells (Capan-1) were treated with AA (10 μM), leading to reduced PAK1 levels and increased PUMA expression. Following AA treatment for 6 h, levels of the indicated proteins were measured by western blot analysis. (C) Cell viability was reduced following AG879 treatment (PAK1 inhibitor), but a synergistic effect with AA was not observed. Following AA and AG879 (5 μM) treatment under SF conditions for 48 h, the MTT assay was performed to measure cell viability. (D) Levels of phosphorylated BAD were reduced in AA-treated cells regardless of PGE2 expression. HCT PUMA-/- cells were treated with AA (10 μM) and PGE2 (10 μM) for 6 h and Okadaic acid (100 nM) for 4 h. (E and F) AA dramatically reduces the protein half-life of PAK1 under SF conditions. Cycloheximide (100 μg/ml) was added to cells in a time-dependent manner to block de novo synthesis. (G) The same result was observed in PC3 cells under SF conditions. (H) The expression of PAK1 was reduced by AA treatment in a dose-dependent manner in HCT116 cells. (I) The effect of AA on the cell death was abolished following BSA treatment. HCT116 cells were treated with AA (10 μM) and 1% BSA. Cell viability was measured by the MTT assay. (J) The AA (10 μM)-mediated reduction in PAK1 expression was recovered following treatment with 1% BSA. (K) The AA-mediated reduction in cell viability was also blocked following BSA treatment. In contrast to AA treatment, celecoxib (Cele) did not induce cell death. After Cele treatment for 48 h, the MTT assay was performed.

cells with AA and measured the PAK1 expression level. As expected, PAK1 expression was undetectable following the addition of 10 μM AA under SF conditions (compared to the 20 μM AA needed to yield the same result under CM conditions) (Figure 3H). To understand the physiological importance, we measured the cell viability of HCT116 cells following AA treatment in SF containing BSA. In fact, albumin can capture AA in blood [30,31], and it can also block SF-induced Smad4 induction and apoptosis [20]. As expected, the addition of 1% BSA into SF media completely blocked AA-mediated cell death (Figure 3I) and PAK1 suppression (Figure 3J). To confirm this result, we measured the viability of A549 cells in BSA-treated media and observed the same effect (Figure 3K). However, celecoxib (Cele), a well-known COX-2 inhibitor, did not induce cell death, indicating that accumulation of the AA intermediate metabolite is not responsible for AA-induced cell death (Figure 3K).

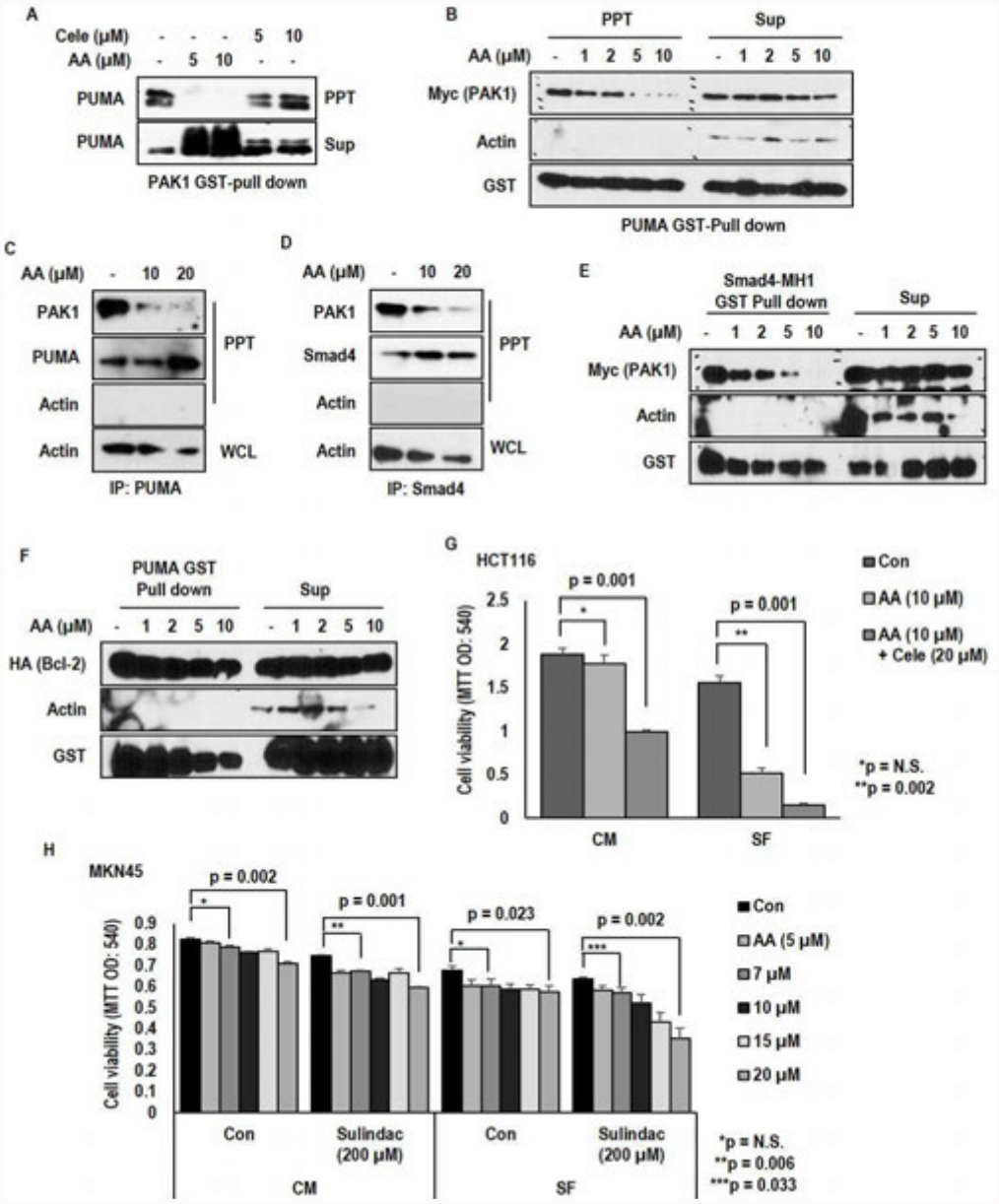

Figure 4: Arachidonic acid blocks the PAK1-PUMA binding and PAK1-Smad4 binding. (A) AA disrupts the binding between PAK1 and PUMA, but celecoxib does not inhibit this interaction. Agarose beads were conjugated to recombinant GST-PAK1 (N-terminal) and then incubated with HEK293 lysates over expressing PUMA. PUMA was then detected by western blot analysis. PPT denotes precipitated proteins, and Sup denotes the supernatant. PUMA levels were measured by western blot analysis. (B) The interaction between PAK1 and PUMA was decreased following AA treatment in a dose-dependent manner. A GST pull-down experiment was performed with agarose beads conjugated to recombinant GST-PUMA. Recombinant PUMA was incubated with whole cell lysate from HEK293 cell which was transfected with PAK1-Myc. PAK1-Myc levels were measured by western blot analysis. (C) The same result was observed by IP analysis with PUMA antibody. Actin was used as a negative control. (D) The binding of Smad4-PAK1 was blocked by AA treatment in a dose-dependent manner. PAK1 and Smad4 were overexpressed in HEK293 cells, and lysates from these cells were used for IP analysis. (E) A similar result was obtained from the GST pull-down assay. HEK293 lysates from PAK1-Myc-transfected cells were incubated with GST-Smad4 (MH1) for 2 h. Actin was used as a negative control. (F) AA did not inhibit the binding of PUMA to Bcl-2. Lysates from HEK293 cells over expressing Bcl2-HA were used for the GST pull-down assay. After incubating the lysates with GST-PUMA for 2 h with the indicated AA concentration, PPT and Sup samples were subjected to SDS-PAGE. Bcl2-HA levels were measured by western blot analysis. (G) Co-treatment of AA (10 μM) and Celecoxib (20 μM) had a synergistic effect on cell viability under both CM and SF conditions. After simultaneous treatment of the two chemicals for 48 h, the MTT assay was performed. (H) Sulindac (COX-2 inhibitor) treatment increases the sensitivity of cells to AA-mediated apoptosis. MKN45 cells were treated with chemicals at various doses, and cells were subjected to the MTT assay following 48 h of treatment.

Arachidonic acid blocks the binding of PAK1 to PUMA and PAK1 to Smad4

To elucidate the mechanism of the AA-mediated effects, we performed a GST pull-down assay with recombinant PAK1 protein and lysates from PUMA-transfected cells. Interestingly, AA inhibited the binding between PAK1 and PUMA, but Cele had no effect on the interaction (Figure 4A). To verify the role of AA in this interaction, we performed a second GST pull-down assay using recombinant PUMA protein and lysates from PAK1-transfected cells; this second pull-down experiment yielded the same result (Figure 4B). An endo-IP with the PUMA antibody also demonstrated the dissociation of PAK1 and PUMA by AA treatment (Figure 4C). These results strongly suggest that AA inhibits the binding between PUMA and PAK1, which could be the main mechanism for AA-induced cell death. PAK1

also interacts with Smad4 [20]; therefore, we investigated whether AA mediates Smad4-PAK1 binding. We found that AA could inhibit the interaction between Smad4 and PAK1 (Figure 4D). To confirm this, we performed a GST pull-down assay using recombinant Smad4 protein and obtained the same result (Figure 4E). However, AA did not inhibit the binding between PUMA and BCL-2 (Figure 4F). These results indicate that the primary target of AA is PAK1 and that AA selectively inhibits PAK1. Because AA can be converted into PGE2 by COX-2, we monitored the combined effect of Cele (COX-2 inhibitor) and AA treatment on cell death. We hypothesized that blocking the conversion of AA to PGE2 may increase the effects of AA, leading to even more cell death. As expected, combined treatment of AA and Cele induced cell death under both CM and SF conditions (Figure 4G). We then evaluated the effect of another COX-2 inhibitor (Sulindac [32]) on AA-induced cell death in MKN45 cells, which are resistant to AA-induced cell death (Figure 1D, 1E) and are known to express high levels of COX-2 [33]. As anticipated, inhibition of COX-2 increased the sensitivity of these cells to AA-induced cell death (Figure 4H). These results suggest that elevated COX-2 levels in some cancer cells may be responsible for their resistance to apoptosis.

Discussion

COX-2 expression is elevated in many types of cancer; therefore, the pro-inflammatory function of PGE2 has been well documented [34]. However, cancer cells have eliminated AA, the precursor of PGE2, despite its usefulness. In fact, AA is an important component of the plasma membrane and is a neurotransmitter [35]. In this study, we postulated that the rapid elimination of AA is required for the survival of cancer cells. To address this hypothesis, we treated cells with AA and found that AA can induce cell death under SF conditions (Figure 1). However, in the presence of serum factors, AA did not induce cell death. We also demonstrated that albumin could inhibit AA-induced cell death (Figure 3I-3K). Thus, we are protected from AA-induced cell death under healthy conditions or when blood is supplied. Conversely, cancer cells are often exposed to serum-deficient conditions (such as avascular regions within a tumor). To overcome this deficiency, cancer cells must eliminate AA *via* COX-2. We observed the sensitization of AA-induced cell death by simultaneously treating cells with COX-2 inhibitors (Figure 4G, 4H).

We also revealed that AA-induced cell death closely resembled SF-induced and Smad4-mediated cell death. In a previous study, we revealed that serum starvation induces apoptosis *via* Smad4-mediated PUMA induction, which is suppressed by PAK1 [20]. In our analysis, we found that AA inhibits the interaction between PAK1 and PUMA and activates PUMA-mediated apoptosis. PUMA-deficient cells are resistant to AA-induced cell death (Figure 2) as well as SF-induced cell death [20].

Smad4 is frequently deleted in human cancers, including pancreatic cancer [36,37]. Therefore, re-activation of PUMA, which is suppressed by PAK1, may be a useful strategy for the treatment of cancer. Since AA can induce cell death independent of Smad4 (Figure 1A, 1B), AA or its modifier may be useful for treatment of Smad4-deficient cancers. However, further studies should be conducted to evaluate whether these potential treatments are viable options.

In summary, AA is a specific inhibitor of PAK1 and can induce apoptosis *via* PUMA activation. In addition, simultaneous treatment of AA and a COX-2 inhibitor can enhance cancer cell death. Our results suggest that AA or a downstream metabolite (one that is resistant to COX-2) could be a useful therapeutic tool for cancer treatment.

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