Biochemical and Biophysical Research Communications **288,** 198–204 (2001)
doi:10.1006/bbrc.2001.5744, available online at http://www.idealibrary.com on IDEAL®

Requirement of c-Jun N-Terminal Kinase for Apoptotic Cell Death Induced by Farnesyltransferase Inhibitor, Farnesylamine, in Human Pancreatic Cancer Cells

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Received September 18, 2001

Farnesyltransferase inhibitors (FTIs) represent a novel class of anticancer drugs and are now in clinical trial. We have previously shown that farnesylamine, synthetic isoprenoid-linked with "amine" which acts as a potent FTI, induces apoptosis in human pancreatic cancer cells through the *ras* signaling cascade. Since the effect of FTI is usually 'cytostatic' rather than 'cytotoxic', we speculated another apoptotic machinery of farnesylamine in addition to the effect of FTI. Farnesylamine induced sustained activation of c-jun N-terminal kinase (JNK), which was not caused by other FTI, FTI-277. Blockage of JNK activity by dominant-negative mutant abrogated the DNA laddering and significantly reduced 'cytotoxic' effect of farnesylamine. Strikingly similar effect on JNK activation and apoptosis was induced by structurally related long-chain fatty amine (LFA), oleylamine, but not by farnesol, an isoprenoid analogue of farnesylamine without "amine." Taken together, apoptosis induction through JNK activation by farnesylamine based on the LFA structure rather than an effect of FTI.

Key Words: **c-jun N-terminal kinase; apoptosis; farnesyltransferase inhibitor; isoprenoid; long-chain fatty amine; pancreatic cancer.**

FTIs developed with the aim of inhibiting the post-translational prenylation and therefore the oncogenic activity of $p21^{ras}$ (1–3). Whereas preclinical proof of principle has been established, the mechanism underlying the antineoplastic properties of FTIs is not fully understood. Previous our study demonstrated that farnesylamine, one of FTI (4), induces apoptosis in human pancreatic cancer cells containing mutated *K-ras* and *K-ras* transformed NIH3T3 (5). However, cells transformed by *K-ras* is usually resistant to FTIs (6–8) because the product of *K-ras* is a more efficient substrate for FTase compared to that of *H-ras* (9) and is alternatively prenylated by geranylgeranyltransferase I in the presence of FTI (10). Furthermore, FTIs usually act in a 'cytostatic' manner against transformed cells, and lack cell toxicity even at concentrations significantly beyond the minimal dose required to inhibit $p21^{ras}$ (11). Recent studies suggest the potential cytotoxicity of FTI in conditions where cells were detached from substratum or deprived from serum (12–15). The exact mechanism of diverse actions of FTI emerges as a question of major interest, and a non-*Ras* target of FTI, such as *Rho* (12), and other distinct pathways (14, 16, 17) are also proposed. These facts led us to speculate that farnesylamine possesses another apoptotic machinery in addition to the inhibition of farnesylation.

Structural feature of farnesylamine is that it contains 15-carbon long-chain lipid, farnesyl isoprenoid, and free amino group, "amine." In human cancer cell lines, growth inhibitory (18, 19) and/or apoptotic effect (20, 21) of isoprenoid has been demonstrated. More recently, potential therapeutic value of farnesyl isoprenoids such as farnesylthiosalicylic acid (22) was documented by *in vivo* animal model using human pancreatic cancer. Furthermore, previous evidence suggests that long-chain fatty amines (LFAs) such as oleylamine and stearylamine are able to inhibit growth of *H-ras* transformed fibroblast, and exert multiple biological activities some of which are shared by farnasylamine (23). In this context, we speculate that the farnesol component or long-chain fatty amine structure of farnesylamine may play a critical role in the apoptotic machinery.

Accumulating evidences suggest that protein kinases, such as mitogen-activated protein kinase

Abbreviations used: FTI, farnesyltransferase inhibitor; JNK, c-jun N-terminal kinase; ERK, extracellular signal-regulated kinase; LFA, long-chain fatty amine.

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0006-291X/01 $35.00

(MAPK) family (24, 25) and phosphatidylinositol 3-kinase (PI3K)/Akt (26, 27), are important regulators of apoptosis. Several studies revealed that c-jun N-terminal kinase (JNK) activation is involved in apoptosis induced by various cellular stresses (28–30) and anti-cancer reagents (31, 32). Despite controversial results are also available (33, 34), much evidence indicates that activation of JNK intimately linked with the apoptotic pathway via phosphorylation of members of Bcl-2 family (35) and cytochrome c release (25). As for mechanism of action of FTI, although there are several reports on the implications of $p21^{ras}$ effectors which promote growth and/or survival signals such as ERK (36, 37) and Akt pathway (12, 14), those of cell death stimulating signals including JNK are not well-elucidated (12).

We have addressed the question of why farnesylamine induces apoptotic cell death in human pancreatic cancer cells containing mutated *K-ras*, which is not usually caused by FTI. To evaluate the molecular basis of the cytotoxicity, we examined the effect of farnesylamine on JNK as a pro-apoptotic signal, in parallel with that of other FTI and structurally related compound, LFA.

MATERIALS AND METHODS

Chemicals and reagents. Farnesylamine was synthesized by TORAY Industries (Tokyo, Japan) as described previously (4, 5). *trans-trans*-Farnesol, fatty amines and their derivatives without amine were purchased from the Fluka Chemie AG (Buchs, Switzerland). FTI-277 was from Calbiochem (La Jolla, CA). Z-VAD-fmk was from Kamiya Biomedical Co. (Seattle, WA), and PD-098059 was from Sigma Chemical Co. (St. Louis, MO).

Cell lines and transfection. Human pancreatic cancer cell lines, PK-1 and PK-9 were provided by Cell Resource Center for Biomedical Research (Tohoku University, Japan) and BxPC-3 was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 containing 10% FBS. PK-1 cells expressing cDNAs encoding dominant-negative JNK (JNK1-APF) (28) was generated using *Trans*IT-LT1 Transfection Reagents (Mirus, Madison, WI). The expression vector, pLNCX-HA-JNK1-APF, was gifted from Dr. Lynn E. Heasley (University of Colorado). After two weeks incubation with 400 μg/ml of Geneticin (Boehringer Mannheim, Indianapolis, IN), three resistant clones were established and the expression of HA-tagged JNK1 mutant proteins was confirmed by Western blotting before the assay (data not shown). NIH3T3 cells were transfected with pZip-ras containing *K-ras* (Val12) (*ras* cell) as previously reported (5).

Quantification of cell viability. The fraction of viable cells was determined using the cell proliferation reagent, WST-1 (Dojin Laboratories, Kumamoto, Japan). This assay is based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in viable cell (38). The statistical significance of differences between groups in each cell lines was assessed by Student's *t* test.

Detection of apoptosis. Low molecular weight chromosomal DNA from cells was purified and subjected to electrophoresis on a 2% agarose gel as described previously (5). Apoptotic cell death was also confirmed by counting cells of DNA content below the G0/G1 peak (sub-G1) using flow cytometry (data not shown) (39).

TABLE 1

Cytotoxic Effect of Farnesylamine, FTI-277 and Farnesol on Human Pancreatic Cancer Cells

		IC 50 (μM)[a]		
Cell lines	K_i-ras	Farnesylamine	FTI-277	Farnesol
PK-1	mutated	21.7 ± 4.3[b]	74.5 ± 7.5[b]	>200
PK-9	mutated	18.3 ± 3.9[b]	87.2 ± 8.3[b]	>200
BxPC-3	wild	28.5 ± 6.7	156.7 ± 10.6	>200

[a] The concentration of reagents required to kill cells by 50% for 24 h exposure measured by WST-1 assay. Values are shown as the mean ± SE of three independent experiments.

[b] Significantly different ($P < 0.05$) from BxPC-3.

In vitro protein kinase assay for JNK, ERK and Akt. MAPKs and Akt activities were measured by the "pull-down" method as described previously (28). In JNK assay, GST-c-Jun fusion protein is used to gather the JNK enzyme before the kinase reaction and phosphorylation of GST-c-Jun was revealed by Western blotting using a phosho-specific c-Jun antibody (New England Biolabs, Inc., Beverly, MA). Cells exposed to reagents were suspended in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride], sonicated, and centrifuged at 10,000 rpm for 2 min at 4°C. Two hundred micrograms of protein was incubated overnight at 4°C with 2 μg of GST-c-Jun conjugated with glutathione sepharose beads. Beads were washed twice with lysis buffer and twice with kinase buffer [25 mM Tris (pH 7.5), 5 mM β-glycerolphosphate, 2 mM dithiothreitol, 0.1 mM Na_3VO_4, and 10 mM $MgCl_2$]. The kinase reaction was initiated by resuspending the pelleted beads in 50 μL of kinase buffer plus 100 μM ATP for 30 min at 30°C and was terminated by adding 25 μL of 3× SDS sample buffer and the samples were then boiled for 5 min. Twenty microliters of the sample were subjected to SDS–PAGE, and immunoblotting was performed using an anti-phosho-c-Jun antibody that recognizes only c-Jun phosphorylated at Ser63, and the phosphorylated-c-Jun was visualized by ECL detection system (Amersham, Buckinghamshire, UK).

Similarly, the activities of immunoprecipitated ERK and Akt were assayed by their ability to phosphorylate recombinant Elk-1 and glycogen synthetase kinase 3β (GSK-3-α), respectively.

Western blot analysis of $p21^{ras}$ processing. Immunoblot analysis was performed to determine the relative amounts of processed and unprocessed $p21^{ras}$ as previously reported (5). Briefly, *K-ras* transformed NIH3T3 cells were treated with farnesylamine and oleylamine for 24 h. Then the cell lysate was immunoprecipitated with anti-*Ras* antibody Y13-259 (Oncogene Science, Uniondale, NY), fractionated by SDS–PAGE, and then probed with Y13-259.

RESULTS

Effect of FTI Does Not Involve in the Apoptotic Mechanism of Farnesylamine

To evaluate whether the apoptotic effect of farnesylamine is reproduced by other FTI, we used one of selective FTIs, FTI-277, which has been shown to induce apoptosis in lines of cancer cells (14). Although high does FTI-277 reduced survival fraction of pancreatic cancer cells (Table 1), apoptosis was not confirmed up to 24 h exposure in normal growth condition (Fig. 1A).

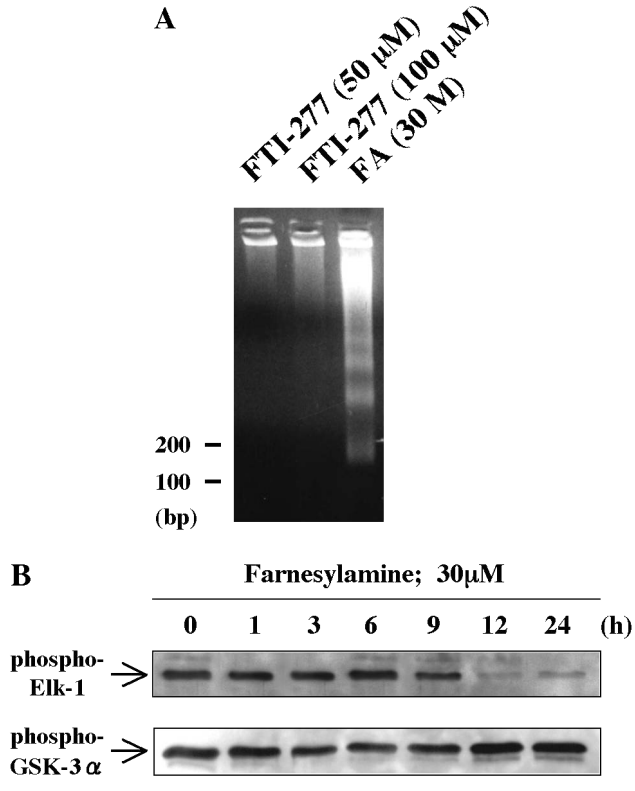

FIG. 1. Apoptotic effect of farnesylamine, but not FTI-277, on human pancreatic cancer cells. (A) PK-1 cells were treated 50 μM FTI-277, 100 μM FTI-277 and 30 μM farnesylamine (FA) for 24 h, and the DNA was isolated and fractionated in a agarose gel electrophoresis as described under Materials and Methods. (B) *In vitro* kinase assay for ERK and Akt. PK-1 cells were treated by 30 μM farnesylamine in exponential growth condition (10% FBS) for the indicated periods. Cell lysates were prepared and kinase activities of ERK and Akt were examined as described under Materials and Methods.

ERK and Akt are the downstream effectors of $p21^{ras}$, both of which evaluated to be influenced by FTI (12, 14, 36, 37). As shown in Fig. 1B, ERK activity was significantly abrogated by farnesylamine, likewise other FTIs (36, 37). However, complete suppression of ERK activity by PD-098059, a specific MEK inhibitor, failed to initiate apoptosis (data not shown), suggesting that the inhibition of ERK is not a primary mechanism in farnesylamine-induced apoptosis. Furthermore, no marked reduction in kinase activity Akt was demonstrated during farnesylamine-induced apoptosis (Fig. 1B). Thus the apoptotic machinery of farnesylamine was explained by neither the effect caused by any other FTI nor direct inhibition of the major downstream effectors of $p21^{ras}$.

Farnesylamine Activates JNK Pathway and Mutated K-ras Promotes the Process

We examined whether activation of stress-induced MAPK, JNK, is associated with the farnesylamine-induced apoptosis as certain kinds of cellular stress (28–30). In PK-1 cells, JNK activity gradually increased to the maximum level up to 9 h in a dose dependent manner, which preceded the initiation of apoptosis (Fig. 2A). JNK activation was not a result of apoptotic event, because caspase-inhibitor, Z-VAD-fmk abolished farnesylamine-induced DNA fragmentation but had no influence on JNK (data not shown). In BxPC-3, containing wild-type *K-ras* (40), the extent of farnesylamine-induced JNK activation was weak in comparison with PK-1 and PK-9 those harbor mutated *K-ras* (39) (Fig. 2B). To confirm the effect of mutated *K-ras* on JNK pathway, we next used NIH3T3, a farnesylamine resistent cell line. Although JNK activation was not induced by farnesylamine in NIH3T3, strong JNK activation was observed in its *K-ras* transfectant (*ras* cells), which was shown to be sensitive to farnesylamine (5) (Fig. 2C).

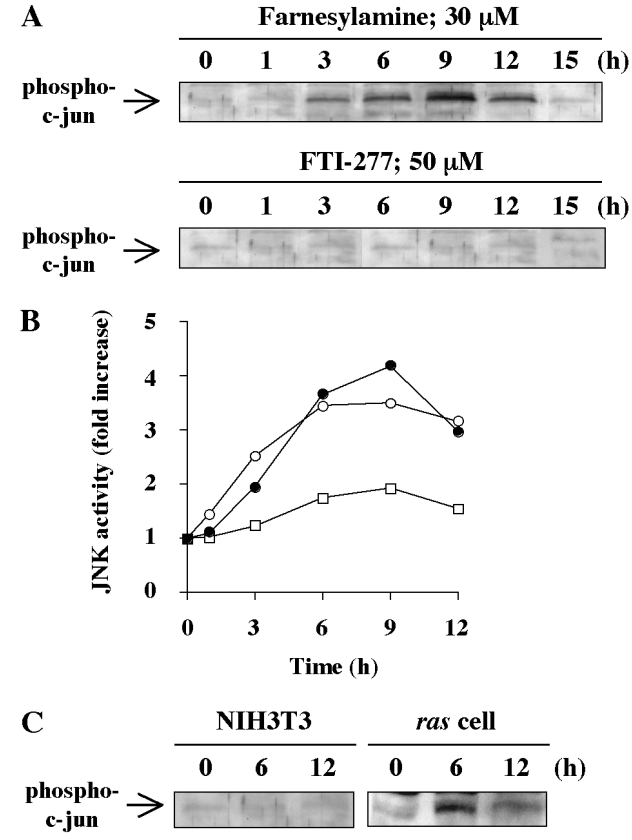

FIG. 2. JNK activation induced by farnesylamine. Total cell lysate from cells treated with farnesylamine was examined using *in vitro* kinase assay as described under Materials and Methods. (A) PK-1 cells treated with 30 μM farnesylamine and 50 μM FTI-277 for the indicated periods in the presence of serum. (B) Farnesylamine-induced JNK activation was monitored for the indicated periods in pancreatic cancer cells containing mutated *K-ras*, PK-1 (●) and PK-9 (○), and in BxPC-3 (□) which has wild-type *K-ras*. The data shown is representative one from three separate experiments. (C) *K-ras* transformed with NIH3T3 (*ras* cells) and its parental cells were treated with 30 μM farnesylamine for the indicated periods.

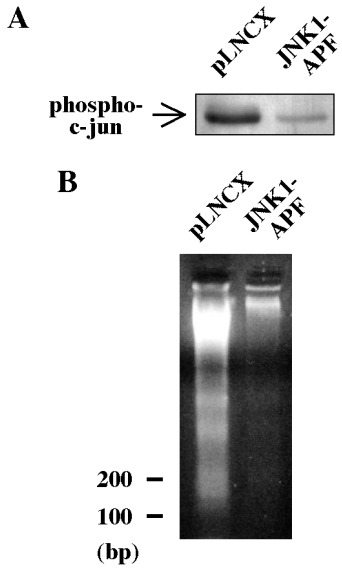

FIG. 3. Effect of dominant negative JNK on farnesylamine induced-apoptosis. (A) After 9 h farnesylamine exposure, JNK assay was performed using total cell lysate from PK-1 cell expressing dominant negative JNK (pLNCX-HA-JNK1-APF) or control vector (pLNCX). The data shown is of representative clone of three independent ones. (B) Each clones were treated by 50 μM farnesylamine for 24 h and DNA laddering was obtained by agarose electrophoresis as described under Materials and Methods.

Equivalent results were not seen after treatment with FTI-277 in the presence of serum (Fig. 2A) suggesting the lack of causal relationship between increased JNK activity and FTI effect of farnasylamine.

JNK Inhibition by Dominant-Negative Mutant (JNK1-APF) Suppresses Farneylamine-Induced Apoptosis

To determine the role of JNK in farnesylamine-induced apoptosis more directly, we used PK-1 cells expressing dominant-negative JNK1, JNK1-APF (28). Expression of the JNK1-APF significantly abrogated the JNK activation induced by farnesylamine (Fig. 3A). JNK1-APF expressing cells required a significantly higher concentration for 50% cell death compared to that for the cells expressing empty vector, pLNCX (51.0 $\pm$ 9.7 μM vs 19.8 $\pm$ 6.8 μM, $P < 0.05$). DNA fragmentation was not observed in PK-1 cells expressing JNK1-APF treated with high dose farnesylamine (Fig. 3B). These results indicate that JNK pathway is indeed involved in the apoptotic process caused by farnesylamine.

Cytotoxic Effect of Long-Chain Fatty Amine on Human Pancreatic Cancer Cells

We examined the association between the apoptotic effect and structure of farnesylamine. Despite the facts that farnesol potently induce apoptosis in human tumor cells (19, 21, 22), cytotoxic effect of farnesol was not obtained in pancreatic cancer cells even at a dose sufficient to induce apoptosis in other cell type malignancies (Table 1). Thus, the effect of farnesylamine was not also simply associated with isoprenoid structure.

Since some biological activities of farnasylamine are shared by LFAs such as oleylamine and stearylamine (23), we speculate that the lipid-linked with "amine" structure plays a critical role in apoptotic effect of farnesylamine. To evaluate the possibility, we next investigated the cytotoxicity of various fatty "amines" on human pancreatic cancer cell lines. LFAs possessing alkyl-chain longer than 12-carbon represented cytotoxicity, but their derivatives without "amine" did not (Table 2). Oleylamine was the most potently cytotoxic among them and the IC50 values in human pancreatic cancer cells was similar to those of farnesylamine. Unlike farnesylamine, which caused an increase in unfarnesylated $p21^{ras}$ (5), oleylamine did not affect prenylation of $p21^{ras}$ suggesting the reagent was not a potent FTI (Fig. 4A). However, likewise farnesylamine, oleylamine induced sustained activation of JNK and cell death with typical DNA laddering (Figs. 4B and 4C), which was completely abrogated by JNK1-APF expression. Oleylamine also induced apoptosis with increased JNK activity in *ras* cells, but not in NIH3T3 (data not shown). Thus, the apoptotic effect of oleylamine through JNK pathway was represented in exactly same form as it was demonstrated by farnesylamine.

DISCUSSION

Over the past years, the mechanism of action of FTIs has been extensively investigated. Contrary to the initial prospect that FTI function by abrogating the $p21^{ras}$-mediated signals, recent studies documented diverse targets and actions of FTI, which seems unlikely relate to the active *Ras*-dependent pathway (12–14, 16,

TABLE 2

Cytotoxic Effect of Long-Chain Fatty Amines on Human Pancreatic Cancer Cell, PK-1

Reagents	Carbon number	IC 50 (μM)[a]
Hexyl alcohol	C6	>300
Hexylamine	C6	>300
Dodecyl alcohol	C12	>300
Dodecylamine	C12	36.0 $\pm$ 5.0
Stearyl alcohol	C18	>300
Stearylamine	C18	25.3 $\pm$ 5.5
Oleyl alcohol	C18:1	>300
Oleylamine	C18:1	14.1 $\pm$ 4.0

[a] The concentration of reagents required to kill cells by 50% (IC50) for 24 hexposure measured using WST-1 assay. Values are shown as the mean $\pm$ SE of three independent experiments.

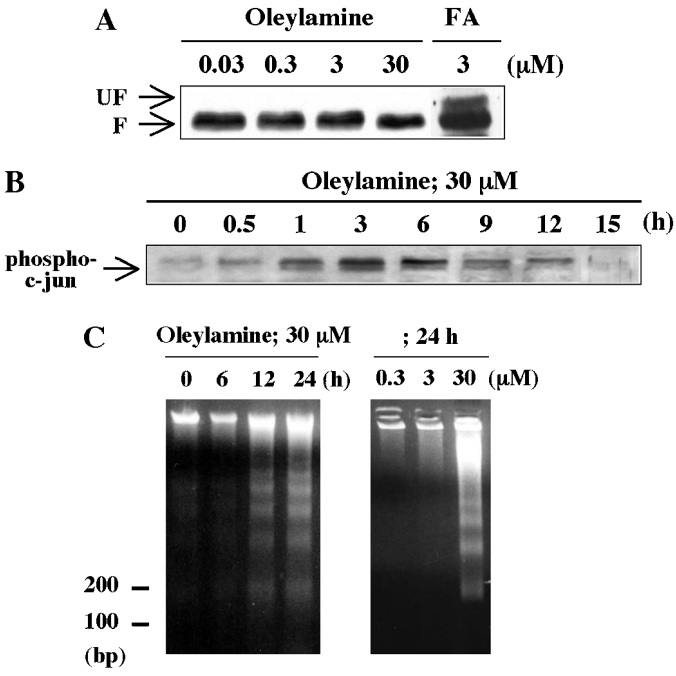

FIG. 4. Apoptotic effect of oleylamine through JNK pathway. (A) Twenty micrograms of total cell lysate from *K-ras* transformed NIH3T3 cells treated with oleylamine and farnesylamine (FA) for 24 h were immunoprecipitated and probed with pan-ras antibody Y13-259. (UF; unfarnesylated $p21^{ras}$, F; farnesylated $p21^{ras}$). (B) PK-1 cells treated with 30 μM oleylamine for the indicated periods, and JNK activity was examined by *in vitro* kinase assay as described under Materials and Methods. (C) DNA isolated from PK-1 cells treated with 30 μM oleylamine for indicated periods and with concentrations of oleylamine for 24 h was isolated and DNA laddering was obtained as described under Materials and Methods.

17). We evaluated a primary role for JNK in the apoptotic process and demonstrated the multi-targeted action of farnesylamine besides inhibition of protein prenylation.

Contrary to our initial expectation, $p21^{ras}$ and its effectors such as ERK (36, 37) and Akt (12, 14) is not critical target of farnesylamine to initiate apoptosis. Although ERK, but not Akt, activity was significantly attenuated by farnesylamine as suggested by previous study (24), it did not play a central role in apoptotic process because complete inhibition ERK by PD-098059 failed to induce apoptosis in pancreatic cancer cell used in the present study. Thus, we cannot conclude that the 'cytotoxic' effect of farnesylamine caused by inhibition of $p21^{ras}$ farnesylation.

In agreement with a pro-apoptotic role of JNK pathway in response to cellular stress (24, 25), our results indicated the essential role of JNK in farnesylamine-induced apoptosis by using dominant negative mutant, JNK1-APF. Although controversial results were also obtained (33, 34), previous evidences still more support the requirement of JNK in apoptosis. First, a persistent activation of JNK due to cellular stress induces apoptosis (28–30), whereas rapid and transient induction caused by survival factors reduces apoptosis (29). Second, the involvement of JNK on apoptosis seems to depend on the concurrent decreased ERK in PC12 cells when nerve growth factor was starved (24) and coordinate activation of JNK with ERK prevent cell from apoptosis (41). Consistent with these findings, our results showed that sustained JNK activation (Fig. 2A) was followed by a decrease in ERK activity (Fig. 1A) during farnesylamine-induced apoptosis.

Our experiments using NIH3T3 indicated *K-ras* promote apoptosis pathway through JNK activation (Fig 2C). In contrast with the evidence on the role of $p21^{ras}$ in cell survival, it has been also suggested that ectopic expression of oncogenic *H-Ras* in murine fibroblasts conferred susceptibility to apoptosis caused by serum starvation or exposure to TNF-α (42,43). Furthermore, several studies indicate the involvement of JNK cascade in the stress stimuli-induced apoptotic process which mediated by deregulated $p21^{ras}$ through an undefined mechanism (44, 45). Those observations obtained by fibroblast system can not be simply applied to human epithelial cancer cells with diverse genetic background (46). However, our results were consistent with $p21^{ras}$-mediated susceptibility to apoptosis in human pancreatic cancer cells, i.e., BxPC-3 harbouring wild-type *K-ras* was significant less sensitive to farnesylamine-induced JNK activation and apoptosis, compared to other cell lines with mutated *K-ras*. These results, along with the fact that JNK is activated in the presence of active *Ras* (28) prompt us to speculate that mutated *K-ras* preferentially sensitize cells to farnesylamine-induced apoptosis by modulating upstream of JNK pathway.

A previous report imply that another class of FTI, L-744,832 activated JNK in cells destined to undergo apoptosis (12). Such an effect on rodent fibroblast was observed only in low-serum condition (0.1% FCS), but not in normal growth condition (10% FCS). However, it is notable that JNK activation by FTIs in human cancer cells is not widely obtained, and it brought up a very important question whether the activation of JNK by the FTI and farnesylamine is related to its inhibitory effect on FTase or the multifunctional aspects. To confirm answer, we used another specific FTI, FTI-277 (14) and concluded that, in normal growth condition, it did not cause JNK activation as well as apoptosis in human pancreatic cancer cells. Furthermore, JNK activation was not induced by lovastatin, another inhibitor of protein prenylation, which potently induced apoptosis in human pancreatic cancer cells (47, 49) (data not shown). Thus, the apoptotic pathway through JNK in the exponential growth condition seems to be also a specific property of farnesylamine rather than a common effect of FTI.

Additional information provided by the present study is that the LFA structure of farnesylamine plays a critical role in apoptosis on human pancreatic cancer

cells. In contrast to other lines of cancer cells (19–21), all pancreatic cancer cell used in the present study was resistance to farnesol-induced apoptosis. However, even in the farnesol sensitive cell lines such as HL-60 (20), association of JNK pathway was not confirmed (unpublished data, H. Ura and Y. Mizukami) and another explanation has been made with respect to the apoptotic machinery of farnesol (21). These results indicate that farnesylamine appears to induce apoptosis through different signaling pathway from farnesol. On the other hand, it is interesting to note that perillyl amine and decaprenylamine, another isoprenoids-linked with "amine," are more growth inhibitory and cytotoxic than those analogues without (19, 20). Furthermore, structurally related compounds LFA including oleylamine have similar biological effect to farnesylamine (23), and those lipid-linked with amine exert their effects via inhibition of protein kinase C (PKC) at least in part (23, 48). In the present study, we do not describe the details about role of PKC, however, it has been shown that, in the presence of activated $p21^{ras}$, loss of PKC function initiates apoptosis in cancer cells (49), and further evidence imply the regulatory role of PKC on NO-induced JNK pathway which cause apoptosis (50). Additional experiments will be required to establish whether such a mechanism account for $p21^{ras}$-mediated JNK pathway in farnesylamine- and oleylamine-induced apoptosis.

In conclusion, farnesylamine induces apoptosis on human pancreatic cancer cells through JNK activation. The activation of JNK pathway seems to be specific for the reagent, not for FTIs in general, and the data presented support the hypothesis that "long-chain fatty amine" component of farnesylamine may be critical for its 'cytotoxic' activity. Further studies directed toward determining the *in vivo* effect of farnesylamine may lead to identification of the novel anti-cancer agent.

ACKNOWLEDGMENTS

We are very grateful to Dr. Lynn E. Heasley (University of Colorado) for the gift of the pLNCX-HA-JNK1-APF. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture, and Technology, Japan.

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