Chelerythrine, a widely used broad-range protein kinase C inhibitor, induces apoptosis in many cell types. In this study, the mechanism of chelerythrine-induced apoptosis in osteosarcoma was investigated.

**Abstract**

**Purpose:** Chelerythrine, a widely used broad-range protein kinase C inhibitor, induces apoptosis in many cell types. In this study, the mechanism of chelerythrine-induced apoptosis in osteosarcoma was investigated.

**Experimental Design:** Signaling pathways activated by chelerythrine in osteosarcoma were detected by Western blots. Impacts of RAF/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase (ERK) kinase (MEK)/ERK MAPK on apoptosis and cell survival were studied using genetic approaches and pharmacologic pathway–specific inhibitors.

**Results:** Osteosarcoma cells underwent apoptosis rapidly after treatment with chelerythrine. Three parallel MAPKs pathways, including the ERKs, c-Jun NH2 kinases, and p38, were activated by chelerythrine in a dose-dependent and time-dependent fashion. For the ERKs, the activation was evident at the earliest time point tested (2 minutes) and sustained for >4 hours. Introduction of a dominant-negative H-RAS mutant (17N) partially attenuated ERK activation and delayed the onset of apoptosis induced by chelerythrine. The ERK activation and apoptotic effects of chelerythrine were greatly abrogated by the pharmaceutical inhibitors of MEK, but not by those of c-Jun NH2 kinase or p38. Moreover, osteosarcoma cells were sensitized to chelerythrine by transient transfection with wild-type MEK1 or constitutively active MEK1 and became resistant with dominant-negative MEK1. Other protein kinase C inhibitors, including GF109203X or Go6976, did not cause ERK activation or apoptosis in the same timeframe tested.

**Conclusion:** In osteosarcoma, chelerythrine-induced apoptosis is mediated through activation of the RAF/MEK/ERK pathway. These findings suggest that activating the ERK MAPK, as opposed to inhibiting it, may be a therapeutic strategy in osteosarcoma.
Opposing the ERK MAPks are cell stress or cytokine-activated JNKs and p38 MAPks, which are often regarded as mediators of apoptosis. Apoptosis is a well-conserved biological process in multicellular organisms, which can be triggered extrinsically through death-related receptors, or intrinsically by damaging the integrity of the mitochondrial membrane (12). Apoptosis is executed by a family of cysteine aspartyl-specific proteases (caspases). The initiators, such as caspase-8 and caspase-9, cleave and activate downstream effectors, caspase-3 and caspase-7, and which in turn cleave a large number of cellular substrates, including the poly(ADP-ribose) polymerase, in an orderly fashion. In a recent study, chelerythrine was reported to be an inhibitor of BCL-XL, a member of the antiapoptotic BCL-2 family involved in stabilizing mitochondrial membrane integrity (13).

Osteosarcoma is a high-grade bone malignancy most commonly seen in children and adolescents (14). Modern treatment of osteosarcoma includes a combination of chemotherapy and surgical procedures. However, 30% to 40% of patients with osteosarcoma can not be cured at the current time, even after an intensive chemotherapy regimen is given (15). Studies including identification of more effective therapeutic agents are needed to further improve the outcome of patients with this disease. In one of our recent studies, chelerythrine was identified to inhibit the proliferation of osteosarcoma cells (16). To further understand the underlying mechanism, this study was therefore initiated.

**Materials and Methods**

**Materials.** Chelerythrine chloride, G66976, and myristoylated PKCζ isoform–specific inhibitor were purchased from BioMol. GF109203X was kindly provided by Dr. Charles Rubin of the Department of Molecular Pharmacology in the Albert Einstein College of Medicine. MEK inhibitor U0126 and PD98059 were purchased from Cell Signaling. All other chemicals were purchased from Sigma. All drugs were reconstituted in DMSO and filter sterilized. All antibodies were purchased from Cell Signaling, unless specified.

**Cell culture.** Osteosarcoma cell lines HOS and U2OS were purchased from American Type Culture Collection. Cells were maintained as a monolayer in MEM-α media supplemented with 10% FCS (Life Technologies). 100 units/mL penicillin, and 3 mg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

**Stable transfection.** A dominant-negative H-RAS mutant (H-RAS-DN, 17N) in a plasmid pcDNA3.0 was a kind gift from Dr. Sakae Tanaka (University of Tokyo; ref. 17). The sequence of the H-RAS-DN construct was confirmed by automated sequencing in this laboratory. Plasmids were transfected into HOS cells with FuGene6 Transfection Reagent (Roche) according to the manufacturer’s instructions. Stable transfection was established by selection with 1,000 μg/ml G418 for 2 wk. Single cell–derived colonies were expanded and tested for H-RAS protein expression by Western blots with an antibody-specific to the COOH terminal of H-RAS (C-20, Santa Cruz Biotechnology). The H-RAS mutant mediated effect was confirmed by comparing the ERK1/2 activation induced by 200 nmol/L 12-O-tetradecanoylphorbol-13-acetate (TPA) and 20 ng/mL epidermal growth factor (Life Technologies) between the H-RAS-DN–transfected HOS sublines and the vector-transfected HOS cells or the parental HOS and U2OS cells.

**Transient transfection.** The 2 × 10⁵ tagged p-MEV plasmids containing human wild-type MEK1 (MEK1-WT), dominant-negative MEK1 mutant (MEK1-DN; K76R, S218A, and S222A), or constitutively active MEK1 (MEK1-CA; Δ32-51, S218E and S222E; ref. 8), were purchased from Biomyx, Inc. HOS cells were plated in 6-cm dishes to reach ~ 50% confluence overnight and then incubated with Fugene6 transfection reagent and respective plasmids for 72 h. Cells were selected with 1,000 μg G418 for 3 d. The remaining G418 resistant cells were pooled, and the expression of MEK1-HA-tag fusion protein was detected by Western blots with a monoclonal antibody specific to HA-tag.

**Viability assay.** Cells were seeded in 96-well plates at a density of 1 × 10⁴ cells per well in serum-containing media overnight to reach ~ 80% to 90% confluence. Chelerythrine was added at a range of concentrations, as indicated, or at 10 μmol/L with pretreatment with the respective inhibitors for 1 h. Equal concentration of solvent (DMSO) was distributed among all the wells. After 2-h incubation with chelerythrine, drug-containing media were aspirated and cells were allowed to recover in fresh media for 2 h. Cell viability was assessed by incubation with 10% Alamar Blue (Biosource) for 4 h. Photo absorbance (A) was measured at wavelengths of 570 and 600 nm, respectively, for each plate with a Benchmark Plus Microplate Spectrophotometer (Bio-Rad). In a fraction of experiments, cell numbers were also measured by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide–based assay with a Cell Growth Determination kit (Sigma) according to the manufacturer’s instructions.

**Proliferation assay.** Cells were seeded in 96-well plates at a density of 4 × 10⁴ cells per well in media containing 5% dialyzed FCS (Life Technologies) overnight. Cell number was measured daily by Alamar Blue assay for four continuous days (days 0-3). Relative cell numbers derived from duplicate plates (24 wells per plate) were plotted as a function of time.

**Cell cycle analysis.** The MEK1 transfected HOS sublines were fixed with 100% ethanol on ice for 30 min and stained with 50 μg/mL propidium iodide, as described previously (18). Cell cycle analysis was done with a Becton Dickinson FACScan in the Flow Cytometry Core Facility in the Albert Einstein College of Medicine. Cell cycle variables were determined with FlowJo analysis software. Experiments were done in duplicate for each transfected subline.

**Western blots.** Total cell lysates were extracted with radioimmununo-precipitation assay buffer. Thirty micrograms of cell lysates were loaded onto a denaturing 10% SDS-PAGE and then transferred to a nitrocellulose membrane. Blotting and stripping (reprobing) were carried out as described previously (16).

**Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assay.** Cells grown in a four-well Lab-tek II chamber slide (Nalge Nunc International) were treated with 10 μmol/L chelerythrine for the indicated durations. Cells were fixed with 4% formaldehyde in PBS at 4°C and paraffinized with 0.2% Triton X-100 in PBS. The terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assay was done with a DeadEnd Fluorometric TUNEL System (Promega) according to manufacturer’s instructions. After washing, cells were then counterstained with 1 μg/mL propidium iodide and were analyzed in the Analytic Imaging Facility of the Albert Einstein College of Medicine using an Olympus IX70 inverted fluorescence microscope with filter set at 520 nm to view the incorporated fluorescein-12-dUTP (green) or at 620 nm to view the propidium staining (red), respectively. Quantification of TUNEL staining was carried out using the ImageJ (Image Processing and Analysis in Java developed by the NIH) software, counting red-stained cells and green-stained cells in three fields in two independent experiments.

**PKC isoforms activity screening.** Cells treated with chelerythrine were screened for PKC activity using a phosphorylated PKC antibody sampler kit (Cell Signaling), which includes phosphorylated-specific antibodies to the conventional PKC isoform α, βII, atypical PKC β, δ, η, and novel PKC ε, λ. The broad-range PKC inhibitor GF109203X (19), the conventional inhibitor Gö6976 (20), or myristoylated PKCζ isoform–specific inhibitors were used compared with chelerythrine.

**PKC kinase assay.** The PKC activation stimulated by 200 nmol/L TPA in the presence of chelerythrine, G66976, or GF109203X, was assayed with a PKC protein kinase assay kit (Calbiochem) according to
the manufacturer’s instructions. The quantification of PKC was determined by measuring photo absorbance (A) at wavelengths of 492 nm relative to that of nonsubstrate control.

**Statistical analysis.** Statistical analysis was done using a software package (SPSS 10.0). A two-tailed Student’s t test was used for all the experiments, and a P < 0.05 was regarded as statistically significant.

**Results**

**Chelerythrine-induced rapid apoptosis in osteosarcoma.** Osteosarcoma cell lines HOS and U2OS treated with chelerythrine for 2 hours rapidly underwent cell death (Fig. 1A). The IC_{50} of chelerythrine was measured by the Alamar Blue assay to be 8.4 and 7.6 μmol/L for HOS and U2OS cells, respectively. Prolonged exposure to 72 hours enhanced the toxicity of chelerythrine. Similar results were obtained using an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide–based assay (data not shown). Cell death by chelerythrine displayed typical morphologic features of apoptosis, such as cell shrinkage and detachment from the plate. This was confirmed by activation of the caspase cascade, including the initiator caspases 8 and 9, the effector caspases 3 and 7, and cleavage of poly(ADP-ribose) polymerase, in a time course after chelerythrine treatment (Fig. 1B). Cell nucleus condensation was prominent after treatment with chelerythrine, as shown by propidium iodide staining (data not shown). The fraction of apoptotic cells after chelerythrine treatment, as indicated by the TUNEL staining, were quantified (Fig. 1C). This was shown to be a rapid process with the caspase activation observed as early as 30 minutes after drug exposure in HOS cells (Fig. 1B). Similar results were observed in U2OS cells (data not shown).

**Chelerythrine-activated MAPK pathways.** It has been reported that the JNKS and p38, but not the ERK1/2 MAPKs, are activated by chelerythrine (5); members of the MAPK family were therefore examined. Contradicting prior results observed in other cell types, phosphorylation of the MEK/ERK1/2 in HOS and U2OS cells was discernible after 30-minute incubation with chelerythrine at the lowest concentration tested (80 nmol/L) and was largely dose-dependent (Fig. 2A). The activation of the MAPK pathway by chelerythrine was also time-dependent (Fig. 2B). The phosphorylated activation of ERK MAPK cascade was evident at the first time point tested (2 minutes) after chelerythrine addition and sustained for >4 hours (Fig. 2B). Activation of p90-ribosomal S6 kinase (p90RSK), a typical downstream kinase of ERK1/2, was also observed (Fig. 2B). In agreement with the previous report (5), MKK4/JNKS and MKK3/p38 MAPK pathways were indeed activated by chelerythrine (Fig. 2C) but at a later time point compared with the ERK cascade (Fig. 2B) in terms of both initiation and development. Among other MAPK members,
ERK5 (the big mitogen activated kinase 1) was also examined, but activation was not evident (data not shown).

**ERK1/2 activation by chelerythrine was partially RAS-dependent.** Among 12 single cell–derived colonies transfected with H-RAS-DN, three clones (C1, C6, and C9) were identified with marked increase of H-RAS expression, as shown in supplementary data (Supplementary Fig. S1A). The H-RAS mutant mediated effect was confirmed by the preferential attenuation of ERK1/2 activation induced by chelerythrine. HOS cells were treated with 10 μM/L chelerythrine for the indicated durations. Activation of the ERK MAPK cascade (B), including RAF1, MEK, ERK1/2, and downstream p90RSK, was detected with the indicated antibodies, respectively. Activation of the MKK4/JNKs and the MMK3/p38 MAPK pathways (C), in response to chelerythrine, were detected with the indicated antibodies and are shown compared with those of the ERK MAPK pathway.

When induced by chelerythrine, the onset of apoptosis, as indicated by the cleavage of caspase-3, was apparently delayed in the H-RAS-DN–transfected HOS sublines compared with the vector-transfected cells (Fig. 3B) at all time points tested (30, 60, and 120 minutes). The viability assay further confirmed that the H-RAS-DN transfected sublines had a survival advantage at early time points of 30 and 60 minutes of chelerythrine treatment (Fig. 3C), but this was not apparent at a later time point of 120 minutes (data not shown).

**ERK1/2 activation and apoptosis by chelerythrine were attenuated by MEK inhibition.** The MEK inhibitor U0126 and PD98059, p38 MAPK inhibitor SB203580, and JNK inhibitor SP600125, were used to identify the signaling pathways involved in the apoptosis induced by chelerythrine. Pretreatment with U0126 or PD98059 dose-dependently blocked ERK1/2 activation by chelerythrine with minor effects on the JNKs and p38 MAPKs activation similarly observed in both...
HOS (Fig. 4A) and U2OS cells (Fig. 4B). Pretreatment with SP600125 or SB203580 blocked chelerythrine-induced c-Jun or ATF-2 activation, respectively (Fig. 4C). Cell death caused by chelerythrine was significantly abrogated by pretreatment with U0126 or PD98059, but not by SP600125 or SB203580 in HOS (Fig. 4C) and U2OS cells (data not shown). Consistently, apoptosis induced by chelerythrine was attenuated by U0126 or PD98059 in a dose-dependent fashion, as indicated by caspase cascade activation (Fig. 4D) and by the TUNEL assay (data not shown), suggesting that activation of the ERK1/2 MAPK is required for apoptosis induced by chelerythrine.

The apoptotic effect of chelerythrine was enhanced by MEK1-WT and MEK1-CA, but abrogated by MEK1-DN. The expression of MEK1-HA-tag fusion protein by transient transfection was confirmed by Western blots with an antibody specific to the HA-tag (Fig. 5A). The molecular size of MEK1-CA detected by the HA-tag antibody was apparently smaller than that of MEK1-DN or MEK1-WT because of a truncation of residues 32-51 (8). As expected, MEK1-CA led to downstream ERK1/2 autophosphorylation in the absence of serum (Fig. 5B, inset). In contrast to a hyperproliferative phenotype observed in epithelial cells or murine fibroblasts transfected with MEK1-CA (8, 21, 22), a significant slower growth rate was observed in HOS cells transfected with the same construct compared with the vector transfected ones (Fig. 5B). Consistently, cell cycle analysis by flow cytometry showed a decreased S-phase population in MEK1-CA, an increased fraction of S-phase population in MEK1-DN, whereas the S-phase population remained unchanged in MEK1-WT (Fig. 5C). However, apoptotic cells represented by the sub-G1 fraction were not apparently observed in any of the sublines. HOS cells were more sensitive to chelerythrine by MEK1-WT (IC_{50}, 3.9 μmol/L; P < 0.01) or MEK1-CA (IC_{50}, 5.0 μmol/L; P < 0.05) transfection and became resistant to it by MEK1-DN transfection (IC_{50}, 8.9 μmol/L; P < 0.01) compared with cells with vector (IC_{50}, 6.9 μmol/L) transfection alone (Fig. 5D). The ERK1/2 activation in response to chelerythrine was found to be enhanced in MEK1-WT and in MEK1-CA and was blunted in MEK1-DN–transfected HOS cells (Fig. 5D, inset).

MAPK activation by chelerythrine was independent of PKC inhibition. Chelerythrine is commonly used as a PKC inhibitor; hence, the similar broad-spectrum PKC inhibitor GF109203X and conventional PKC inhibitor Go6976 were used to challenge cells. However, unlike chelerythrine, GF109203X and Go6976 did not induce ERK1/2 activation or

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**Fig. 3.** A, effects of H-RAS-DN on ERK1/2 activation induced by chelerythrine. Stably transfected HOS cells were treated with chelerythrine at the indicated concentrations for 60 min. ERK1/2 activation in the H-RAS-DN transfected sublines (C1, C6, and C9) was assayed by SDS-PAGE immunoblotting and compared with the vector-transfected HOS cells. The same blot was stripped and reprobed with a MEK antibody as a loading control. The H-RAS-DN mutant expression in stably transfected HOS sublines was further confirmed with the C-20 antibody. B, effects of the H-RAS-DN on the onset of apoptosis induced by chelerythrine. Cells were treated with 10 μmol/L chelerythrine for the indicated durations. The onset of apoptosis was assessed by SDS-PAGE immunoblotting probed with a cleaved caspase-3 antibody. The same blot was stripped and reprobed with a β-actin antibody as a loading control. C, viability assay. H-RAS-DN transfected HOS sublines and the vector-transfected HOS cells were treated with chelerythrine at the indicated concentrations for 30 min (left), 60 min (right), or 120 min (data not shown). Cell viability obtained from two independent experiments is shown as the mean and SE. ** and *** indicate a statistical significance of P < 0.01 and P < 0.001, respectively, as determined by a two-tailed t test.
apoptosis in HOS cells (Fig. 6A) or in U2OS cells (data not shown). The effect of chelerythrine on PKC activity was examined on most of the PKC isoforms. In the absence of chelerythrine, the basal phosphorylation level of the PKCs was fairly low (data not shown) consistent with our previous study (16). After chelerythrine exposure, no apparent change of phosphorylation was observed among all isoforms examined, except the novel PKCζ, in which an increase of phosphorylated activation was observed and seemed to be both dose-dependent (Supplementary Fig. S2A) and time-dependent (data not shown). Pretreatment with myristoylated PKCζ isoform specific inhibitor blocked the activation of PKCζ by chelerythrine (Supplementary Fig. S2B) but had no effects on ERK1/2 activation (Supplementary Fig. S2B) or apoptosis (data not shown) induced by chelerythrine. Myristoylated PKCζ isoform specific inhibitor alone did not activate ERK1/2 MAPK (data not shown). These results suggest ERK1/2 activation is not downstream of the PKCζ activation induced by chelerythrine in osteosarcoma. When PKC was alternatively activated by TPA, pretreatment with GF109203X and Gö6976 blocked the activity of PKC, whereas no apparent effects were observed in cells pretreated with chelerythrine (Fig. 6B).

Discussions

In osteosarcoma cells, chelerythrine concomitantly activates three parallel MAPK signaling cascades, including ERK1/2, JNKs, and p38 MAPKs were detected by SDS-PAGE immunoblotting with the indicated antibodies. C. effects of MAPK inhibitors on viability of cells treated with chelerythrine. HOS cells were pretreated with solvent (DMSO) only, the MEK inhibitor U0126 and PD98059 (PD), p38 MAPK inhibitor SB203580 (SB), or JNK inhibitor SP600125 (SP), at the indicated concentrations for 1 h. Cells were then treated with 10 μmol/L chelerythrine (CHE) for 2 h. Relative cell viability of the treated group compared with that of the nontreated control is presented as mean and SE derived from three independent experiments. * and ** indicate a statistical significance of P < 0.01 and P < 0.001, respectively, as determined by a two-tailed t test. The effect of SP600125 on downstream c-Jun activation and that of SB203580 on ATF-2 activation were assayed using SDS-PAGE immunoblotting with the indicated antibodies. D. effects of MEK inhibitors on apoptosis induced by chelerythrine. HOS cells were pretreated with U0126 or PD98059 at the indicated concentrations for 1 h. Cells were then treated with 10 μmol/L chelerythrine for 2 h. The effects of MEK inhibition on apoptosis induced by chelerythrine were determined by SDS-PAGE immunoblotting with the indicated antibodies, including caspase-3, caspase-7, and poly(ADP-ribose) polymerase. Cells treated with solvent (DMSO) only were used as a negative control and those treated with chelerythrine only were used as a positive control for apoptosis. Dose-dependent inhibition by U0126 and PD98059 on ERK activation was further confirmed by SDS-PAGE immunoblotting. The same blot was stripped and reprobed with a β-actin antibody as a loading control. Representative blots from two independent experiments are shown.
at such doses, chelerythrine did not have detectable effects on cell viability for either a short term (2 hours) or a long term (72 hours), as was observed in previous studies (5, 6). Therefore, the activation of MAPKs by chelerythrine is unlikely a result of cellular stress in osteosarcoma. The rapid activation of the MAPK pathways after chelerythrine addition resembles a typical activation cascade of a receptor tyrosine kinase induced by its biological ligand. This is also supported by activation of the downstream target kinase p90RSK by chelerythrine. Chelerythrine is reactive to cysteine (4, 5), which is abundant in the conserved extracellular domains of receptor tyrosine kinases. It is possible that chelerythrine may cause dimerization or oligomerization of a yet to be identified receptor(s) and subsequent ligand-independent activation. RAS-dependent ERK1/2 activation by receptor tyrosine kinase or RAS-independent activation through PKCs was reported previously (17, 26–28). In the current study, chelerythrine-induced ERK1/2 activation seemed to be partially RAS-dependent, suggesting heterogeneous receptor activation. Furthermore, the activation of ERK1/2 induced by chelerythrine was MEK-dependent, as indicated by ERK attenuation by the MEK inhibition through both pharmacologic and genetic approaches. It is noted that the structurally related analogues, sanguinarine and chelerythrine, were identified as inhibitors of MAPK phosphatase-1 (29), which dephosphorylates MAPKs. Therefore, this mechanism is unlikely to be responsible for the activation of MAPKK (MEK, MKK3, and MKK4) and MAPKKK (RAF1) upstream in the signaling cascade, as observed in the current study.

In previous studies, activation of JNKs and p38 MAPKs secondary to oxidative stress was reported to be the mechanism for apoptosis by chelerythrine (5, 6). However, apoptosis induced by direct administration of H₂O₂ was a much slower process than that induced by chelerythrine (6). It is noted that, in previous studies, the apoptotic effect of chelerythrine was only abrogated by excess amount of cysteine-containing antioxidants, such as N-acetyl-L-cysteine, glutathione, and DTT, but not by similar sulfur-containing ones, such as DMSO (5, 6). This suggests that chelerythrine was possibly neutralized by cysteines of these antioxidants extracellularly (chemical antagonism; refs. 5, 6). Moreover, although JNKs or p38 MAPKs were indeed activated by chelerythrine in the current study, their inhibitors did not have an apparent effect on the apoptosis induced. Therefore, activation of JNKs and p38 MAPKs is unlikely to be the underlying mechanism of apoptosis induced by chelerythrine. Instead, multiple lines of evidence identified in this study suggest that chelerythrine-induced apoptosis is mediated by activation of the RAF/MEK/ERK MAPK pathway: (a) chelerythrine activated the RAF/MEK/ERK pathway and induced apoptosis dose-dependently and time-dependently;
(b) MEK1-specific inhibitors U0126 and PD98059 attenuated ERK1/2 activation and apoptosis induced by chelerythrine; (c) overexpression of MEK-WT, which relays and amplifies the upstream signals, or MEK1-CA overexpression, which may lower the external signal threshold required, enhanced ERK1/2 activation and the apoptotic effects of chelerythrine; (d) MEK-DN transfection blunted ERK1/2 activation and cell death caused by chelerythrine; and (e) H-RAS-DN partially attenuated ERK1/2 activation and delayed the onset of apoptosis by chelerythrine. Although the detailed mechanism underlying the apoptosis induced by chelerythrine is unclear, the current study suggests an extrinsic pathway, as indicated by the activation of caspase 8. Givens the known extensive cross-talk between the receptor-mediated pathway and the mitochondrial pathway (12), the BCL-xL inhibition and cytochrome c release observed in previous studies (6, 13), as well as caspase 9 activation observed previously (6) and in this study, are likely the results of cross-activation by the extrinsic pathway. MEK inhibition abrogated caspase activation, suggesting that ERK1/2 activation is required and may be involved in the initiation stage of apoptosis upstream of caspase activation. The H-RAS-DN transfected cells, although delaying the onset of apoptosis, were still susceptible to chelerythrine. This may not be surprising. Unlike MEK, which is regarded as the dedicated kinase for ERK, RAS serves as a switch for an array of signaling pathways in addition to RAF/MEK/ERK. A possibility is that some prosurvival signals mediated by RAS might be disrupted by the RAS mutants collaterally. Alternatively, clonal selection generated during transfection may bypass and mask the requirement of RAS/RAF/MEK-mediated signaling for cell proliferation. This should be investigated in a future study. In addition, it is interesting to note that, at very high concentrations of chelerythrine (50 μmol/L), the activation of ERK/MEK seemed to be decreased (Fig. 2A). The underlying mechanism is not clear. In myocytes, apoptosis was induced only when the concentration of chelerythrine was between 6 and 30 μmol/L, as reported in a previous study (6). This seemed to be in parallel with the range of dose-dependent ERK/MEK activation by chelerythrine observed in the current study. This also suggests that dose of chelerythrine may need to be carefully controlled to observe the maximal MEK activity.

Chelerythrine is commonly used as a broad-range PKC inhibitor. In the current study, chelerythrine did not show apparent inhibition on TPA-induced PKC activity compared with other broad-range or traditional PKC inhibitors (Fig. 6B). This was similarly observed in previous studies (4, 5, 30). Interestingly, other PKC inhibitors did not cause ERK activation or apoptosis in the timeframe tested, suggesting that PKC inhibition was not associated with ERK activation or apoptosis induced by chelerythrine. The involvement of PKCζ activation in the effects of chelerythrine is not clear, although our data showed ERK activation is unlikely to be a result of it.

The ERK1/2 MAPK is among the most common dysregulated pathways identified in tumors, as indicated by frequently observed activating mutations in RAS or BRAF in cancers, particularly in colon cancer and malignant melanoma (10). Overwhelming lines of evidence in the literature point to an oncogenic, mitogenic, and prosurvival role for this pathway. In contrast, some evidence suggests that the ERK MAPKs pathway may mediate differentiation in PC12 cells and osteoblasts (9, 18, 31, 32). ERK1/2 activation has been reported to be involved in apoptosis or growth arrest in some tissue types (18, 33, 34). To date, little information is available regarding the role of this pathway in osteosarcoma. A gene knockdown approach of ERK1/2 in osteosarcoma cells might provide specific information to better understand its role. In this study, transient transfection of MEK1-CA alone, which resulted in ERK1/2 autophosphorylation and a delay of cell proliferation, perhaps through cell cycle alteration, did not induce apoptosis directly (Fig. 5C). This is perhaps due to relatively weak enzyme activity compared with the fully activated wild-type MEK or compensation by other signaling pathways. This suggests that although ERK1/2 activation is required for apoptosis induced by chelerythrine, it may not be sufficient. It was reported previously that in an estrogen-response RAF inducible system, wild-type MEK was activated and led to strong ERK1/2 activation, and instead of stimulating proliferation, growth arrest was observed (18). Particularly interestingly, it was reported that enforced RAS-CA expression resulted in permanent growth arrest in osteosarcoma, which was similarly released by MEK inhibition (31, 35). Recently, it was shown that activation of ERK MAPKs was essential for apoptotic effects of a variety of chemotherapeutic agents, including etoposide, doxorubicin, gemcitabine, cisplatin, and carboplatin (36–39). Taken together, these results suggest that activating ERK MAPK, rather than inhibiting...
it, may be of therapeutic merit in osteosarcoma, and this will be investigated further in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

Activation of the RAF/Mitogen-Activated Protein/Extracellular Signal-Regulated Kinase Kinase/Extracellular Signal-Regulated Kinase Pathway Mediates Apoptosis Induced by Chelerythrine in Osteosarcoma

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