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MEK inhibition enhances bleomycin A5-induced apoptosis in an oral cancer cell line: signaling mechanisms and therapeutic opportunities

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BACKGROUND: Bleomycin A5 is an anti-neoplastic glycoprotein antibiotic used for the treatment of various cancers. Previous work has shown that bleomycin A5 exerts its apoptotic effects on tumor cells. This was to study the signal transduction pathways that might exert the apoptotic effects of bleomycin A5 on tumor cells, as well as to examine the possibility of lower dosing of such drug in combinative treatment with other compounds *in vitro*.

METHODS: Bleomycin A5 was applied on a human oral epidermoid carcinoma cell line, human oral epidermoid carcinoma (KB), and the apoptotic activity was determined by the presence of DNA fragmentation and 4,6-diamidino-2-phenylindole (DAPI) nuclear staining. The signal transduction pathway was measured through Western blotting and *in vitro* kinase assay.

RESULTS: The apoptotic effect was associated with the sustained activation of c-Jun N-terminal kinases (JNK) and the inhibition of extracellular signal-regulated kinases1 (ERK1) and -2 activities, suggesting that JNK plays a positive role in the death process. ERK1 and -2 might exert a protection pathway from cell death. Here, it was determined that a combination treatment of bleomycin A5 and the MAP kinase-ERK kinase (MEK) inhibitor, PD98059, could lead to enhanced apoptosis. The activities of ERK1 and -2 are required for cell survival signaling using stable cell clones expressing MEK1. Upon bleomycin A5 treatment, cells expressing MEK1 exhibited significant delays in the onset of apoptosis, where the presence of MEK1 inhibitor enhanced cell death. Moreover, the increased activity of ERK1 and -2 coincided with cell survival. The survival signals exerted by MEK1 most likely result from the activation of ERK1 and -2.

CONCLUSIONS: The apoptosis enhancement through such combinative treatment *in vitro* has revealed new therapeutic opportunities and elucidated mechanisms contributing to the efficacy of existing anti-cancer treatments.

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Introduction

Cell death is as essential as cell proliferation for the development and maintenance of healthy tissues in mammalian development (1–3). Dysregulated apoptosis contributes to many pathologies, including tumor promotion, autoimmune and immunodeficiency diseases, and neurodegenerative disorders (4). Growth factors play important roles in modulating such processes by transmitting their signals through specific receptor-generated signal transduction pathways, which might affect the cell survival or death decision. Therefore, signal transduction pathways that trigger apoptosis are of intense interest. The mitogenic activated protein kinases (MAPK) family is an essential part of the signal transduction machinery in signal transmissions from cell surface receptors and environmental stimulation (5). At least three groups of MAPK subfamilies have been identified in mammals: (i) the extracellular signal-regulated kinases (ERK) (6); (ii) the c-Jun N-terminal kinases (JNK, also called stress-activated protein kinase) (7); and (iii) the p38 MAPK (8). In most cases, ERK contributes to the cell response to signals generated from growth factors through an Ras-dependent pathway, which might play a major role in regulating cell growth and differentiation (9). Moreover, the activation of the ERK pathway was shown to be necessary and sufficient to induce transformation in mouse muscles fibroblast cell line (NIH3T3) cells (10). The activated ERK is believed to confer a survival signal to cells (11, 12). In contrast, JNK and p38 MAPK are activated by environmental stresses, such as UV, osmotic pressure, heat shock, lipopolysaccharide, and protein synthesis inhibitors (13–15). The JNK and p38 MAPK are also activated by cell treatment with pro-inflammatory cytokines, including interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α)

(16). Their activation is most frequently associated with the induction of apoptosis (17). The activation of MAPKs, therefore, contributes to complex regulatory cellular events including growth, differentiation, survival, and death. Among them, the JNK signaling pathway is essential for neuronal apoptosis in response to exocytotoxic stress (18, 19). Therefore, the therapeutic induction of apoptosis and the general pharmacology of apoptosis have become subjects of great interest and the 'magic bullet' to eliminate cancer.

Bleomycin A5 is a glycopeptide antibiotic used clinically in the treatment of various cancers. Both bleomycin and its derivative peplomycin are used as chemotherapeutic agents in the treatment of oral cancers (20, 21). The cytotoxic effect of bleomycin A5 is believed to result from its ability to bind iron, activate oxygen, and cleave DNA and RNA (22). The detailed mechanism by which such nucleic acid damage triggers apoptosis remains unclear. Understanding the molecular basis of bleomycin A5-mediated apoptosis could lead to strategies resulting in improved therapeutic benefits. The rational molecular mechanism by which bleomycin A5 might be activated is the MAPK subfamilies. In this report, bleomycin A5 effects on tumor cell apoptosis were tested. It was determined that bleomycin A5 is able to prolong the activation of the JNK as well as the suppression of the activities of ERK1 and -2 in a tumor cell line, KB. In this study, KB cells were used to evaluate the role of the MEK-ERK pathway in apoptosis suppression and the role of sustained JNK activation involved in its ability to promote apoptosis. The MEK1-expressing KB cells have demonstrated their survival activity against the bleomycin A5 treatment. The effect of a combination of bleomycin A5 and MEK1 inhibitor, PD98059, were tested on tumor cell apoptosis. This combinative treatment of the inhibitor for MEK1 and bleomycin A5 enhanced programmed cell death in tumor cells and has suggested a new cancer therapy.

Materials and methods

Cell culture

All experiments described in this paper were performed with KB cells. KB cells were derived from an epidermal carcinoma of the mouth. KB cells were grown in Dulbecco's modified minimal essential medium (Life Technologies Inc., Rockville, MD, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and containing 100 μg/ml streptomycin and 100 units/ml penicillin at 37°C in 5% CO_2.

Reagents

Bleomycin A5 was purchased from Taihe Pharmaceutical Co, Tianjin. PD98059 was purchased from Cell Signaling. The MEK1 inhibitor (PD98059; Cell Signaling Technology, Beverly, MA, USA) has been shown to act *in vivo* as a strong inhibitor of MEK1 activation and the MAP kinase cascade (23).

JNK kinase assay

Protein kinase assays were carried out using a fusion protein between glutathione S-transferase (GST) and c-Jun (amino acids 1–79) as a substrate. The GST-Jun fusion proteins were bound to glutathione sepharose beads and incubated for 15 min on ice with the cellular extract that contains JNK, in the presence of kinase buffer (20 mM Hepes, pH 7.6, 1 mM EGTA, 1 mM dithiothreitol, 2 mM MgCl, 2 mM MnCl, 5 mM NaF, 1 mM NaVO, and 50 mM NaCl). The beads were pelleted and thoroughly washed with PBST (150 mM NaCl, 16 mM sodium phosphate, pH 7.5, 1% Triton X-100, 2 mM EDTA, 0.1% MeOH, 0.2 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine) before they were incubated with [γ-^{32}P] ATP (50 cpm/fmol) in the presence of kinase buffer. These steps were undertaken to ensure that c-Jun phosphorylation was carried out by JNK, which is known to exhibit high affinity to this portion of c-Jun under these conditions (24). Following extensive washing, the phosphorylated GST-Jun was boiled in SDS sample buffer and the eluted proteins were run on a 15% SDS–PAGE. The gel was dried and phosphorylation of the Jun substrate was determined by autoradiography.

Establishment of MEK1-expressing cells

KB cells were transfected with 10 μg of purified pBabe puro, pBabe mitogen-activated protein kinase kinase (MAPKK), or pBabe MAPKKE217/E221 DNA following $CaPO_4$ protocol. Forty-eight hours after transfection, cells were split into a 0.25-μg/ml puromycin selection medium. Selection medium was changed every other day. The selection colonies were pooled 2 weeks after selection.

Western blot analysis

Immunoblot analysis was performed with the anti-phospho p44/42, anti-p44/42, anti-phospho p38, and anti-p38 (Cell Signaling). Basically, cells were harvested in lysis buffer (50 mM Tris–HCl, pH 8.0/250 mM NaCl/1% NP-40, 2 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 ng/ml leupeptin, 50 mM NaF, and 1 mM sodium orthovanadate. Total proteins were then separated on SDS–PAGE and specific protein bands visualized with an ECL chemiluminescent detection system (Amersham Pharmacia Biotech, Little Chalfont, UK) was performed as described.

Evaluation of apoptosis

Cells grown on 60-mm plates were washed three times with phosphate-buffered saline and fixed with 4% paraformaldehyde. Fixed cells were again washed three times with phosphate-buffered saline and stained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/ml; Sigma Chemical Co., St. Louis, MO, USA) for 30 min. Stained cells were examined with a fluorescence microscope to identify apoptotic cells.

DNA fragmentation assay

The method of apoptotic DNA fragment analysis developed by Herrmann and colleagues (25) was described in detail previously. Briefly, cells were harvested, washed, and pelleted by centrifugation. The cell pellets were then treated with lysis buffer (1% NP-40, 20 mM EDTA, in 50 mM Tris–HCl, pH 7.5) for 10 s. After centrifugation, the supernatants were brought to 1% SDS and treated with RNase A (final concentration at 5 μg/ml, Sigma) at 56°C for 2 h followed by digestion with proteinase K (final concentration at 2.5 μg/ml; Sigma) at 37°C for at least 2 h. Apoptotic DNA fragments were separated by electrophoresis in 2% agarose gels.

Results

Bleomycin A5 is a glycopeptide antibiotic isolated from *Streptomyces pingyangensisn* and is used clinically in the treatment of various cancers. The cytotoxic effect of bleomycin A5 is believed to be associated with the induction of apoptosis (26, 27). Whether the apoptosis of KB cells induced by the treatment of bleomycin A5 was confirmed with a characteristic apoptosis assay on oligonucleosomal fragmentation of DNA of KB cells in a dose-dependent manner. Other features characteristic of apoptosis, including cell shrinkage, membrane blebbing, and chromatin condensation, were also present in cells treated with bleomycin A5 (data not shown).

To establish a causative relation between the apoptosis induced by bleomycin A5 and the signal transduction pathways among MAP kinase family, KB cells were treated with bleomycin A5 at concentrations of 0.5 and 5 mg/ml for 1 h. Cells were harvested and lysates were assayed for phosphorylation of p44/42MAPK(ERK1/2) and p38MAPK using Western blotting. Increasing the bleomycin A5 dosage resulted in a reduction of p44/42MAPK phosphorylation (Fig. 1A). In contrast, bleomycin A5 treatment led to no change in the phosphorylation of p38MAPK (Fig. 1A). Furthermore, cells maintained the same amount of p38MAPK in response to bleomycin A5 treatment (Fig. 1A). These results demonstrate that bleomycin A5-induced KB cells' apoptosis coincides with the dephosphorylation of p44/42MAPK.

Among MAPKs, the JNK signaling pathway has been implicated in apoptosis when cells are exposed to many apoptotic agents (13, 28). Therefore, the JNK activities in the apoptotic KB cells were examined. KB cells were treated with 0.5 and 5 mg/ml of bleomycin A5 and cell extracts were collected 1 h after the bleomycin A5 treatment. An *in vitro* kinase assay was performed to test the JNK activities at this point. The activation of JNK was observed at 0.5 mg/ml, as well as at 5 mg/ml in the presence of bleomycin A5 in a KB cell culture (Fig. 1B). These results indicated that bleomycin A5 induced the activation of a JNK pathway and inactivated the ERK signaling in a dose-dependent manner.

To test the possible influence on MAPKs activity following prolonged KB cell culture exposure to bleomycin A5, KB cells were tested with 5 mg/ml of bleomycin A5 and cell extracts were collected at different time points. An *in vitro* kinase assay was performed to test the JNK activities at these points. The induction of JNK activities could be detected in KB cells following bleomycin A5 treatment as early as at 1 h and further enhanced at 4 h and sustained at the highest

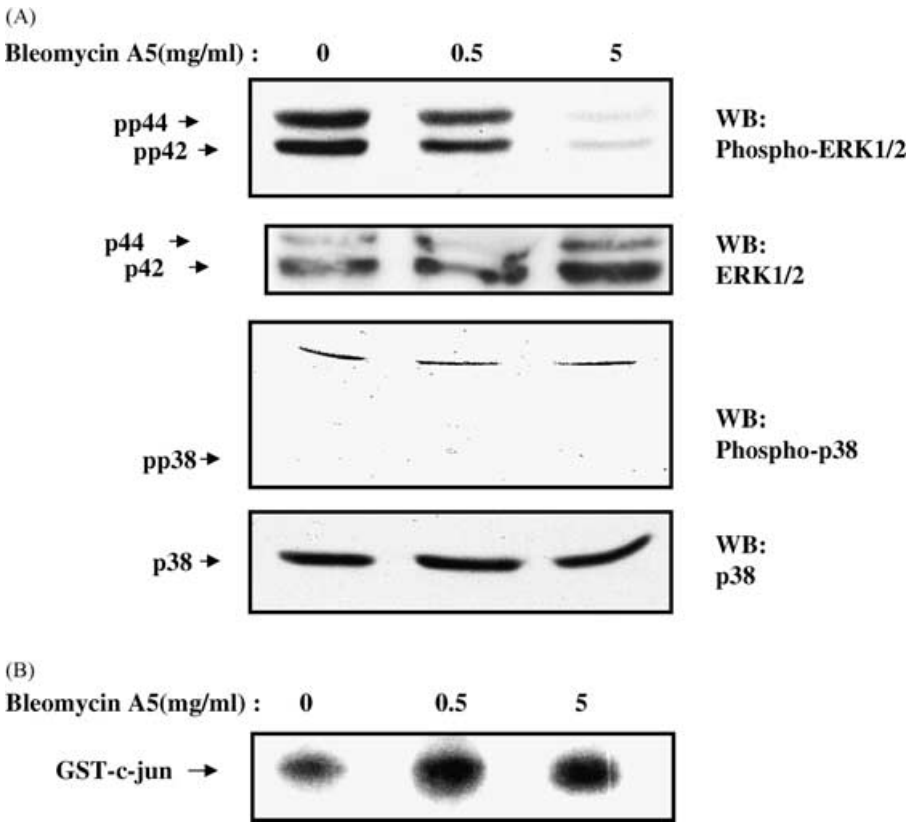

Figure 1 Dose-dependent effects of bleomycin A5 on MAP kinases. (A) KB cells were treated with 0.5 or 5 mg/ml bleomycin A5 for 1 h, after which cell lysates were assessed for ERK or p38 activation using Western blotting with an anti-phospho-ERK or anti-phospho-p38 antibody. For the internal control, blot was probed with an anti-p38 antibody. (B) Solid phase *in vitro* activation of JNK by bleomycin A5. Bacterial expressed GST-c-jun (1–79) proteins were immobilized through binding to glutathione sepharose 4B. This substrate was incubated with the indicated cell lysate (20 μg) with or without bleomycin A5 treatment in the presence of [γ-^{32}P] ATP. The kinase reaction was carried out at 25°C for 15 min, and the phosphorylated substrates were separated by SDS–PAGE and detected by autoradiography. These experiments were performed three times; representative data are shown.

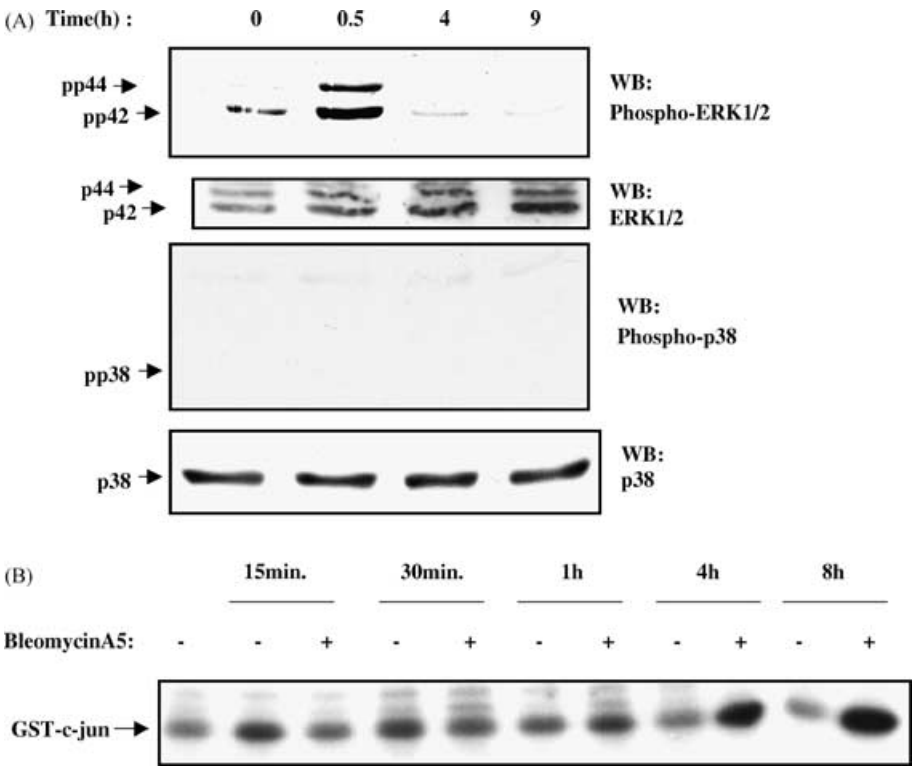

Figure 2 Time-dependent effects of bleomycin A5 on MAP kinases. (A) KB cells were treated with 5 mg/ml bleomycin A5 for the indicated time, after which cell lysates were assessed for ERK or p38 activation using Western blotting with an anti-phospho-ERK or anti-phospho-p38 antibody. For the internal control, blot was probed with an anti-p38 antibody. (B) The activation of JNK was sustained following the bleomycin A5 treatment. Solid phase *in vitro* activation of JNK was performed following the treatment with 5 mg/ml bleomycin A5 for the indicated time. The results showed a significant sustained JNK activation. Results shown are representative of at least two independent experiments.

activities through 8 h (Fig. 2B). In the control experiment, transient induction of the JNK activities reached maximum within 30 min and returned to the basal level within 1 h (Fig. 2B). The induction of the JNK activities in the control experiment was because of the existence of serum. Moreover, the transient induction to serum for the JNK activity was abolished by the presence of bleomycin A5 in the KB cell culture. As per the above results, bleomycin A5 not only activated the JNK pathway but also suppressed ERK signaling. The phosphorylation status of ERK1/2 was tested through Western blot analysis following the bleomycin A5 treatment at different time points. The presence of bleomycin A5 dramatically decreases p44/p42 phosphorylation by 4 h. Phosphorylation status is increased at 30 min because of the serum effects on the KB cells (Fig. 2A). The cell extracts were tested for bleomycin A5 effects on the other signal molecule, p38MAPK. No increase in the phosphorylation of this protein and no change in the total amount of p38MAPK (Fig. 2A) were found. The results indicate that bleomycin A5 induces apoptosis in KB cells and might exert its effect on KB cells for apoptosis through the internal signal transduction machinery of the MAPK family. The resultant JNK activation and reduction in the phosphorylation status of ERK1/2 were significantly found 2 h earlier than oligonucleosomal DNA fragmentation, which was first detected at 6 h after 5 mg/ml of bleomycin A5 was treated on KB cells (data not shown). This result indicated that the activation of the JNK signaling and inactivation of the ERK signaling might play a role in apoptosis after bleomycin A5 treatment.

In this study, it was demonstrated that bleomycin A5 induces apoptosis in KB cells through signals to two MAPK pathways. The induction of the JNK pathways and suppression of ERK pathways occurred in parallel with the apoptosis in tumor cells through bleomycin A5 treatment. Recently, polypeptide growth factors have played a critical role in suppressing cell death using the ERK pathway (29). The JNK signaling pathway is essential for certain cell types in response to exocytotoxic stress (30). Therefore, the hypothesis of promoting tumor cell apoptosis through a mechanism that might decrease the basal activity of ERK1/2 and sustain the activation of JNK pharmacologically was tested. A compound, PD98059, shown to act *in vivo* as a highly selective inhibitor of MEK1 activation and the ERK1/2 pathways, was used in concert with bleomycin A5 in the KB cell culture. KB cells were pre-treated with or without 10 μM of PD98059 for 12 h and then treated with concentrations of 125, 500, 1250, 2500, and 5000 μg/ml for 9 h. Oligonucleosomal DNA fragmentation was collected to determine the apoptotic cells. Cells pre-treated with PD98059 were observed to markedly enhance bleomycin A5-induced apoptosis through oligonucleosomal DNA fragmentation (Fig. 3C) and DAPI nuclear staining assay (Fig. 3D; Table 1). To ascertain that KB cells were pre-treated with PD98059 without significantly affecting the activities of JNK, KB cells were manipulated under

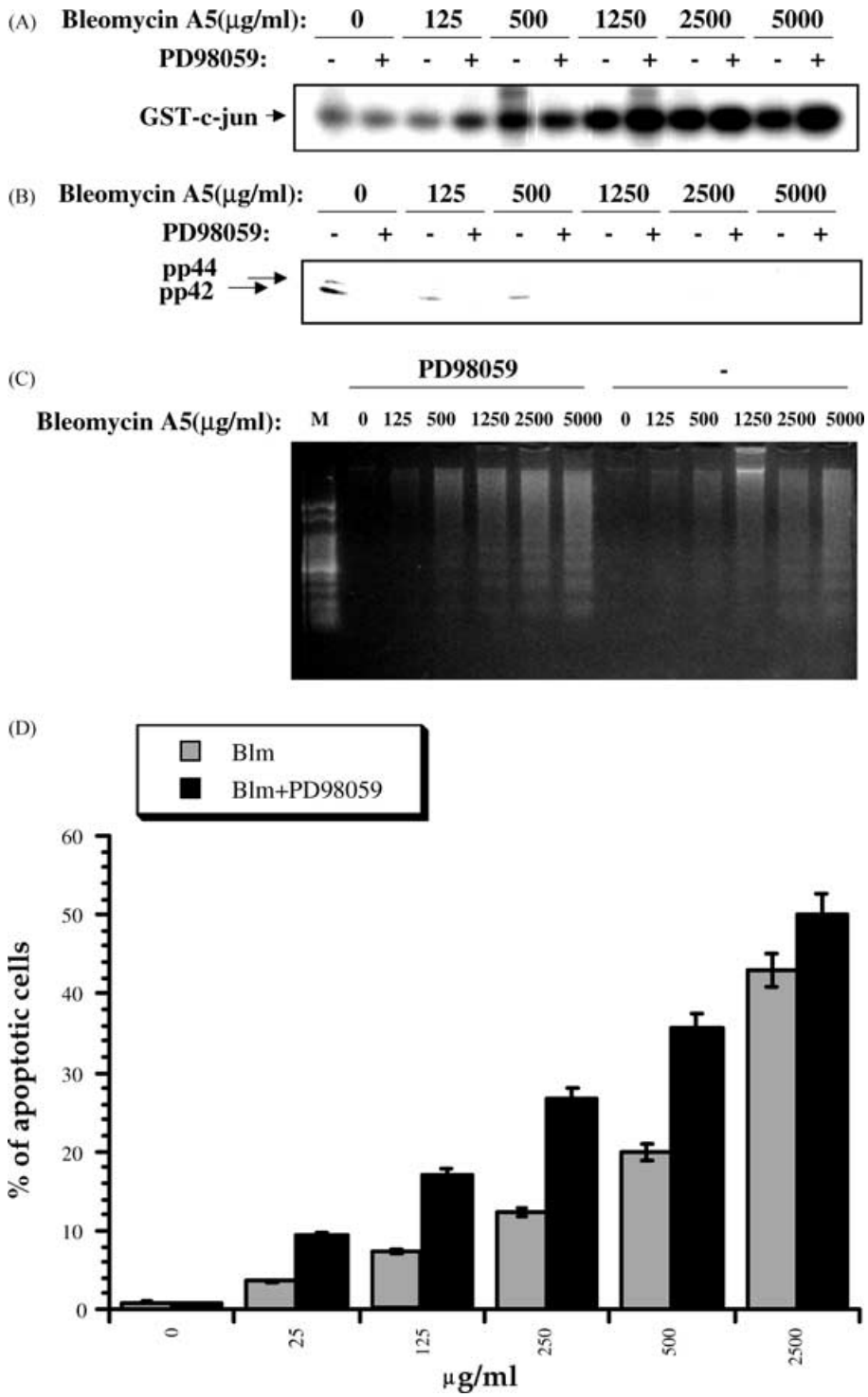

Figure 3 Analysis of bleomycin A5 and MEK1 inhibitor on KB cell death and their effects on JNK and ERK signalings. (A) The JNK activities were not further enhanced after the combined treatment of bleomycin A5 and PD98059 in KB cells. KB cells were pre-treated with or without 10 μM PD98059 for 12 h and then added with or without the combination of 10 μM PD98059 and the indicated dose of bleomycin A5 for 4 h, and JNK kinase activity was assayed as described in the section under Materials and methods. (B) ERK activities were analyzed from the same lysates using Western blot analysis using an anti-phospho-ERK antibody. (C) KB cells were treated as described above, and after 9 h, soluble DNA was extracted for DNA fragmentation assay. (D) Bleomycin A5 and PD98059 caused enhanced KB cell death. KB cells were treated as described above, and after 24 h, the percentage of apoptotic cells was determined by counting the number of cells containing condensed or fragmented nuclei relative to the total number of cells following nuclear staining with DAPI. Results shown are representative of at least four independent experiments.

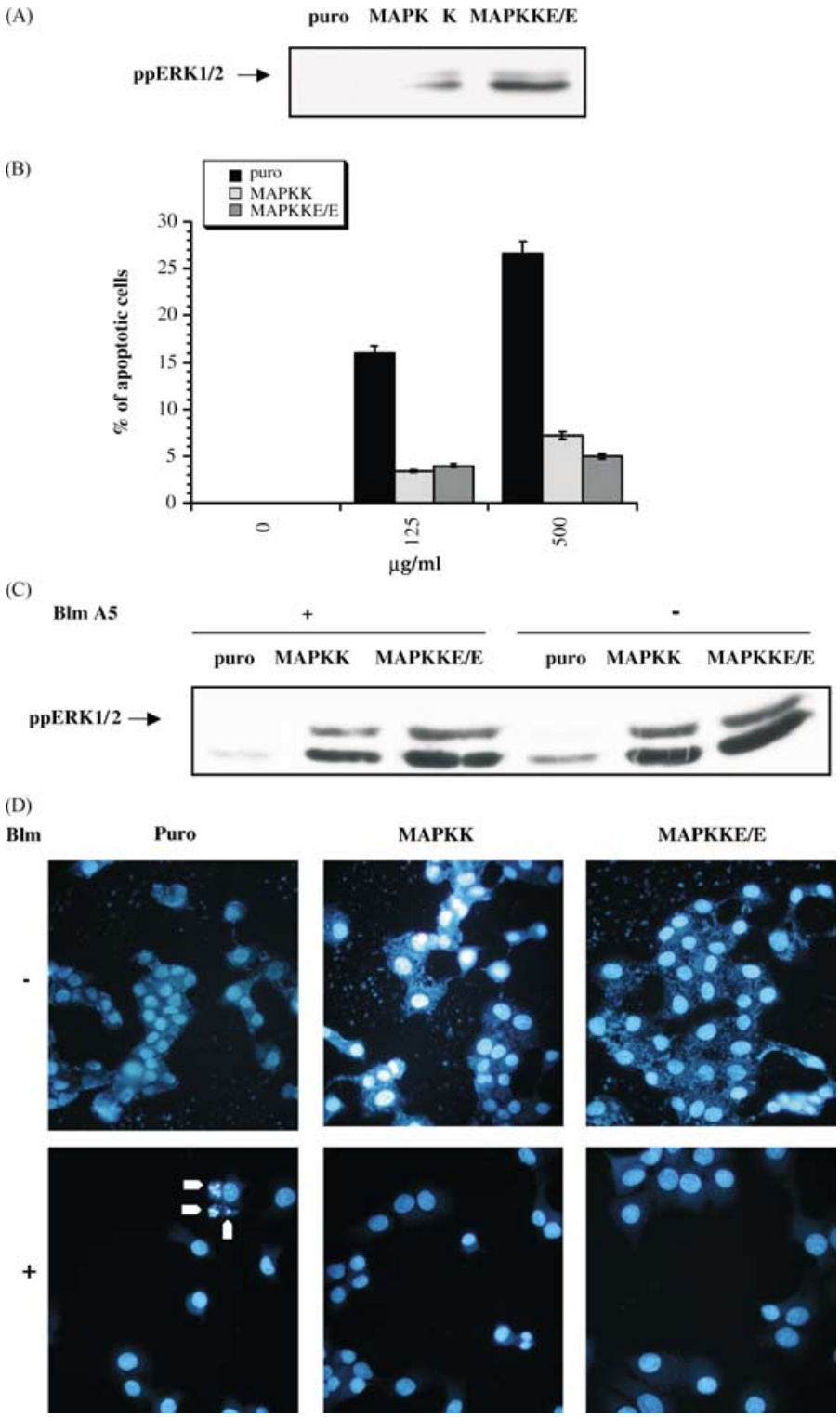

Figure 4 Overexpression of MEK1 prolong activates ERK1/2 and prevents apoptosis of bleomycin A5-treated KB cells. KB cells were stably transfected with a plasmid driving the expression of either the wild type or constitutively activated MEK1. (A) ERK activity in transfected cells was determined using Western blot analysis using an anti-phospho-ERK antibody. (B) Effect of MEK1 expression on bleomycin A5-induced apoptosis in KB cells. Apoptotic cells were assayed by nuclear DAPI staining 24 h following indicated concentration of bleomycin A5 treatment. (C) Prolong activation of ERK1/2 was coincident with the delay of cell death in MEK1-expressing KB cells following 500 mg/ml bleomycin A5 treatment. The activation status of ERK1/2 was analyzed using an anti-phospho-ERK antibody. (D) DAPI staining of control or MEK1-expressing KB cells treated with 500 μg/ml bleomycin A5 for 24 h. Nuclei of apoptotic cells are fragmented and condensed, indicated by arrows. These experiments were performed three times; representative data are shown.

Table 1 Combination treatment with bleomycin A5 and MEK inhibitor PD98059

Treatment	*Vector*	*MAPKK*	*MAPKKE/E*
Control	0	0	0
Bleomycin	10.7 ± 0.31	4.9 ± 0.17	6.25 ± 0.24
PD	0	0	0
PD + bleomycin	28.4 ± 1.24	13.7 ± 0.64	23.6 ± 1.27

Ectopic expression of either the wild type or constitutively activated MAPKK cells treated with a combination of 10 μM PD98059 (PD) and 500 μg/ml bleomycin for 24 h. The percentage of apoptotic cells was determined by counting the numbers of cells containing condensed or fragmented nuclei relative to the total number of cells following nuclear staining with DAPI. The data represented are the mean ± SE.

conditions previously stated. Cell lysates were harvested at 4 h after bleomycin A5 treatment, and an *in vitro* kinase assay was performed to determine the JNK activities. JNK activities did not significantly change between the control and the PD98059 pre-treated group (Fig. 3A). Conversely, the ERK1/2 pathways were dramatically diminished following PD98059 treatment (Fig. 3B). These results indicated that ERK and JNK pathways might exert opposite effects on cell survival in KB cells, where the long-term activation of JNK might be pro-apoptotic, and ERK activity was seen to contribute a protective effect.

To ascertain the role of ERK activities correlated with the suppression of apoptosis, KB cells expressing either the wild type or constitutively activated versions of the MAPKK, MEK1, were generated. Both genes in the retroviral expression vector pBabe puro were then used to transfect parental KB cells. After the puromycin selection, Western blotting analysis verified selection expression of the MEK1 transgene in the resulting cell pools to increase the phosphorylation status of ERK1/2 (Fig. 4A). The question if KB cells with either the wild type or constitutively activated MEK1 conferred a survival advantage upon bleomycin A5 treatment was then addressed. KB puro cells carrying only the vector, or KB MAPKK, and KB MAPKKE/E expressing the wild type and the constitutively activated MEK1, respectively, were treated with bleomycin A5. As shown in Fig. 4(B, D), 24 h after treatment, dramatic cell death occurred among the control cells. This was less pronounced in both the wild type and constitutively activated MEK1 cells. To test whether the expression of MEK1 resulted in the activation ERK1 and -2 coincides with apoptosis suppression by MEK1, antibodies were employed to detect the phosphorylations of ERK1 and -2. As shown in Fig. 4(C), the phosphorylation of ERK1 and -2 was higher than that in controls and still maintained after bleomycin A5 treatment, indicating that ERK activation might be essential for apoptosis suppression by MEK1. To provide further evidence for the requirement of MEK1 for the survival of MEK1-expressing KB cells, cells were pre-treated with PD98059, followed by a combination treatment of the inhibitor and bleomycin A5. Table 1 summarizes the enhanced apoptosis observed in the control and ectopic MEK1-expressing KB cells with PD98059 and bleomycin A5, confirming that the protective effect observed in these cells depended upon signaling activated by MEK1. These results indicate a powerful molecular and rational drug targeting. The combination therapy with MEK1 inhibitor and bleomycin A5 might allow the usage of lower dosage and less toxicity of drugs to enhance tumor killing *in vivo*.

Discussion

The signals that direct cells toward the apoptotic pathway following bleomycin A5 treatment are not known. Here, it shows that bleomycin A5 affects MAP kinases. Identifying the MAPK activities among the kinase family is therefore critically important for our understanding of mitotic and apoptotic regulation. Bleomycin A5-induced apoptosis in KB cells is mediated through the activation of JNK and suppression of ERK1/2. JNK signaling is triggered by UV irradiation, osmotic stress and in response to cytokines and growth factors. JNK has recently been implicated as a major apoptotic pathway following DNA damage and TNF-α and in response to the Fas ligand (31). The bleomycin A5 treatment on KB cells may therefore lead to DNA damage and trigger a potent apoptotic response. The most pronounced effect is JNK activation. The results show that sustained JNK activation is closely associated with apoptosis induction. This result is consistent with the hypothesis that sustained JNK activation leads to apoptosis (32). In the control experiment, only transient JNK activation occurred because of the serum effects, and no apoptosis was induced. In addition to the activation of JNK, bleomycin A5 characteristically causes ERK1/2 hypophosphorylation. The study showed that a potent inhibitor for MEK, PD98059, is able to potentiate the bleomycin A5 apoptotic effect on KB cells. The effect of PD98059 has been proved to reduce the *in vivo* invasiveness of a human squamous cell carcinoma cell line (UM-SCC-1; 33), and is thought to result from blockage of urinary-type plasminogen activator (uPA) expression (34). Their data indicated that ERK1/2 activation contributes to the invasiveness of oral carcinoma. Conversely, the MEK1 expressing cells are resistant to the bleomycin A5 treatment. These results demonstrated an inactivation of the MEK-ERK pathway in response to bleomycin A5. Moreover, MEK1 is able to interfere with the bleomycin A5 apoptotic effect on KB cells. Whether the inactivation of ERK1/2 after bleomycin A5 treatment is simply mediated afterwards, the inactivation of MEK1 or other upstream targets will require further investigation.

Based on ERK1/2 and JNK differential activity results from bleomycin A5-treated KB cells, a potential cancer therapy using a combination of bleomycin A5 and the MEK1 inhibitor PD98059 on KB cells is presented. These two pharmacologic agents, bleomycin A5 and PD98059, caused the activation of JNK and the inactivation of ERK1/2. This combination treatment might result in changes in the dynamic balance between JNK and ERK1/2 to determine cell fate. This finding suggests a potential improvement in cancer therapy through lower drug doses and enhanced *in vivo* tumor killing.

Over the past 2 years, increasing evidence has been accumulated suggesting that an MEK-ERK pathway may play a role in apoptosis regulation (35). Using the chemical inhibitor PD98059 of MEK with bleomycin A5, an essential role for MEK1 in apoptosis suppression is demonstrated. The presence of PD98059 alone is not sufficient to trigger

the apoptosis in this study. However, Shakibaei et al. (36) showed that the inhibition of MEK alone through its inhibitor U0126 induced apoptosis in human chondrocytes. To provide further evidence for the effect of MEK1 on apoptotic inhibition, both the wild type and constitutively activated MEK1 expression cells were generated. MEK1-expressing cells are much more resistant to apoptosis than pBabe puro empty vector controls using bleomycin A5 treatment. The anti-apoptotic effect was correlated with the increased ERK signaling activity. It is therefore suggested that the survival response signaled by MEK1 is most likely the result of ERK1/2. von Gise et al. (12) also showed that the expression of a constitutively active form of erk2 was able to protect NIH3T3 cells against doxorubicin-induced cell death. According to these studies, the apoptosis suppression suggested a role for MEK-ERK-dependent signals for this process.

The important aspect of these findings is that the apoptosis induced by bleomycin A5 results in two signaling cascades that are jointly required for the achievement of certain biological responses. These findings suggest the potential biologic JNK and MEK-ERK functions. The employment of bleomycin A5 and the MEK inhibitor in combination enhances apoptosis, which might be a potential anti-cancer therapy by allowing lower drug doses and less toxicity during *in vivo* chemotherapy.

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