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Etoposide-induced activation of c-*jun* N-terminal kinase (JNK) correlates with drug-induced apoptosis in salivary gland acinar cells

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Abstract

We have examined the ability of etoposide to induce apoptosis in two recently established rat salivary acinar cell lines. Etoposide induced apoptosis in the parotid C5 cell line as evidenced by the appearance of cytoplasmic blebbing and nuclear condensation, DNA fragmentation and cleavage of PARP. Etoposide also induced activation of c-*jun* N-terminal kinase (JNK) in parotid C5 cells by 4 h after treatment, with maximal activation at 8–10 h. Coincident with activation of JNK, the amount of activated ERK1 and ERK2 decreased in etoposide-treated parotid C5 cells. In contrast to the parotid C5 cells, the vast majority of submandibular C6 cells appeared to be resistant to etoposide-induced apoptosis. Likewise, activation of JNKs was not observed in etoposide-treated submandibular C6 cells, and the amount of activated ERK1 and ERK2 decreased only slightly. Etoposide treatment of either cell line had no effect upon the activation of p38. Treatment of the parotid C5 cells with Z-VAD-FMK, a caspase inhibitor, inhibited etoposide-induced activation of JNK and DNA fragmentation. These data suggest that etoposide may induce apoptosis in parotid C5 cells by activating JNKs and suppressing the activation of ERKs, thus creating an imbalance in these two signaling pathways.

Keywords: Salivary acinar cells; apoptosis; JNK; c-*jun* N-terminal kinase; etoposide; salivary gland

Abbreviations: EGF, epidermal growth factor; ERK, extracellular regulated kinase; JIP1, JNK interacting protein-1; JNK, c-*jun*-N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MKP, MAP kinase phosphatase; PARP, poly-(ADP) ribose polymerase; SRF, serum response factor; TCF, ternary complex factor; ZVAD, N-benzyloxycarbonyl-Val-Ala-Asp-(O-methyl)-CH_2F

Introduction

Failure of the salivary gland causes many medical problems. Salivary gland hypofunction, which leads to dry mouth or xerostomia, can occur as the indirect result of long-term drug treatment of the elderly,[1] X-irradiation of patients with head and neck tumors,[2] and chemotherapy of patients with a diverse variety of tumors and/or leukemia.[3] Patients with Sjögren's syndrome suffer from a loss of salivary gland function as a result of an autoimmune response directed against this tissue.[4] In each of these cases the loss of salivary gland cells appears to result from apoptosis or programmed cell death.

As a means to understand the molecular pathways regulating apoptosis in salivary acinar cells, we have examined the activation of mitogen-activated protein (MAP) kinase family members in response to treatment of two recently established salivary acinar cell lines with etoposide, a chemotherapeutic drug also known as VP-16. The MAP kinase family contains three different subfamilies: the extracellular regulated kinase (ERK) family composed of ERK1 and ERK2;[5] the JNK/SAPK family comprised of at least three different genes that each encode multiple proteins formed by alternative splicing;[6–11] and the p38/*HOG1* family.[12–17] All MAP kinases are proline-directed serine-threonine protein kinases that are activated by the phosphorylation of both threonine and tyrosine residues in a Thr-X-Tyr motif in the activation loop that lies in close proximity to the ATP and substrate binding sites. We focused upon this family of signaling molecules because of their central role in signaling processes, because pathways regulating these enzymes are reasonably well defined, and because of the evidence that these enzymes can contribute to either mitogenic (anti-apoptotic) or pro-apoptotic responses in different cell types. Our results demonstrate that etoposide is a potent inducer of apoptosis in parotid C5 cells, while the submandibular C6 cells appear to be largely resistant to etoposide. Activation of the JNK pathway and inhibition of the ERK1 and ERK2 pathways correlates with the induction of apoptosis by etoposide in the parotid C5 cells, suggesting that apoptosis may result from an imbalance in these signaling pathways.

Results

Etoposide induces apoptosis of the parotid C5, but not the submandibular C6 cell lines

Apoptosis occurs in a series of well defined steps that involve specific biochemical changes to the cell. Subconfluent cultures of the parotid C5 and submandibular C6 cell lines were treated with 100 μM etoposide for varying periods of

time and the appearance of nuclear condensation was examined (Figure 1). After 6 h of treatment with 100 μM etopside there was a small but detectable increase in the number of condensed parotid C5 cells compared to untreated cultures (Figure 1, panel A *versus* panel B), which further increased at 12 h (Figure 1, panel C) and was virtually complete by 24 h (Figure 1, panel D *versus* panel E). In contrast, treatment of the submandibular C6 cell line with 100 μM etoposide did not result in dramatic cell death (Figure 1, panels G–I). Etoposide treatment appears to cause both cell types to become flatter and larger.

A dose response study was also conducted to compare the effects of etoposide upon the parotid C5 and submandibular C6 cell lines. There was an increase in the number of condensed C5 cells treated with 10 or more μM etoposide, with a maximum effect at 100 μM etoposide (data not shown). Submandibular C6 cells were unaffected even by 200 μM etoposide (data not shown). We examined the appearance of a DNA ladder in etoposide-treated cells, the release of histone-associated low molecular weight DNA from the nucleus of treated cells, and the activation of caspase-3 following etoposide-treatment (Figure 2). As shown in Figure 2A, using the DNA ladder assay we could detect DNA fragmentation in the parotid C5, but not the submandibular C6 cells 18 h after treatment with etoposide (Figure 2A, lane 3 *versus* 6). When twice as much DNA from etoposide-treated submandibular cells was analyzed, a small amount of fragmented DNA could be detected at 18 h in these cells (data not shown).

We also examined the appearance of histone-associated low molecular weight DNA in the cytoplasm of parotid C5 and submandibular C6 cells treated with 100 μM etoposide for 18 h. This assay can provide a more quantitative measurement of DNA fragmentation than the traditional DNA ladder assay; it also revealed a significant difference in the sensitivities of the two cell lines (Figure 2B). As little as 5 μM etoposide caused a significant increase in histone-associated DNA fragments present in the cytoplasm in parotid C5 cells, with a maximal effect at 50 μM etoposide. The submandibular C6 cell line was significantly less sensitive to etoposide; requiring and, at 100 μM etoposide producing an effect equivalent to that seen with 10 μM etoposide in the parotid C5 cells (Figure 2B).

Numerous apoptotic stimuli induce the activation of caspase-3 (also known as CPP32, apopain, and Yama).[18–20] The activation of caspase-3 was examined with a commercial kit in which the cleavage of Ac-DEVD-pNA (N-acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline) is detected by a colorimetric assay. There is a substantial difference between these two cell lines in regard to caspase-3 activation (Figure 2C). There was a linear increase in caspase-3 activation in the parotid C5 cells until 8 h, with a total increase of 10–15-fold increase by 18 h (Figure 2C). A small but detectable increase in caspase-3 activity was detected in the C6 cell line, which returned to basal

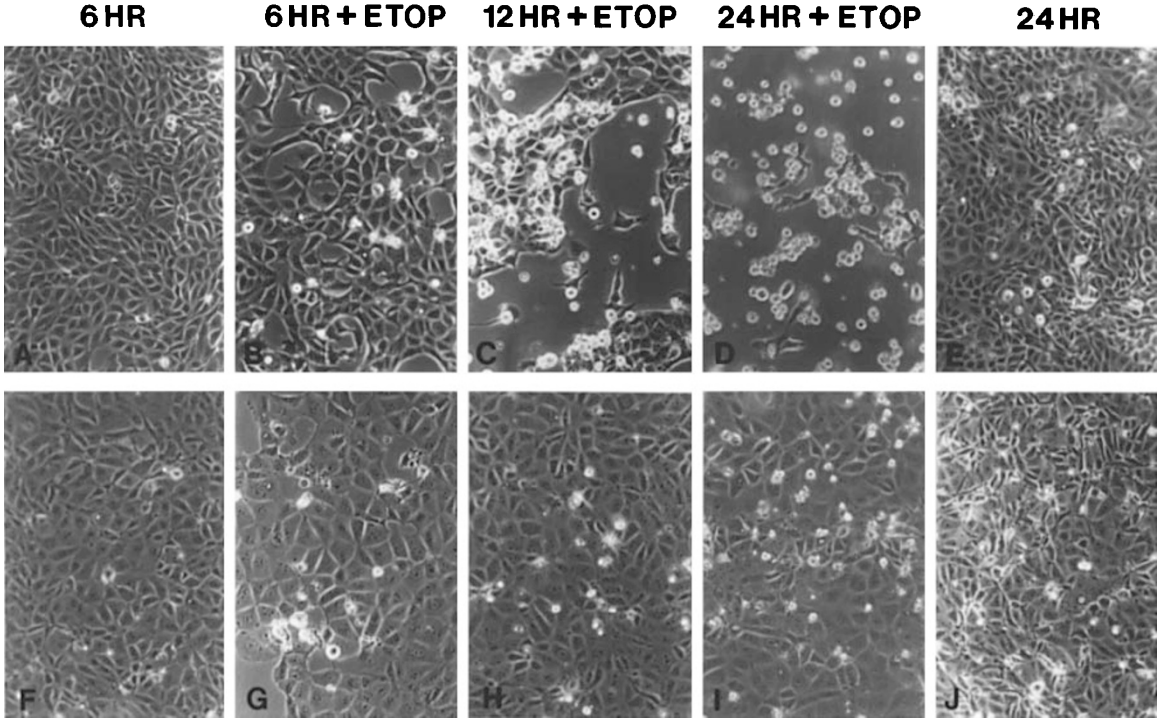

Figure 1 Etoposide induces changes in cellular morphology consistent with apoptosis in the parotid C5 cell line, but not in the submandibular C6 cell line. Subconfluent monolayers of the parotid C5 (**A**–**E**) and the submandibular C6 (**F**–**J**) cell lines were treated with 100 μM etoposide for varying periods of time and the morphology of the cells examined microscopically. Untreated cells are shown at 6 h (**A** and **F**), and at 24 h (**E** and **J**). Cells treated with 100 μM etoposide are shown at 6 h (**B** and **G**), 12 h (**C** and **H**), and 24 h (**D** and **I**)

levels after 8 h. By 18 h, the difference between C5 and C6 was more than 30-fold.

Etoposide induced activation of JNK in the parotid C5 cell line

Different members of the MAP kinase family are activated in response to stimulation with mitogenic or apoptotic agents. We examined the activation of different MAP kinase family members in response to treatment with etoposide. In the controls, both the parotid C5 and submandibular C6 cell lines contained ERK1, ERK2, JNK1, JNK2, and p38 protein kinases at equivalent levels, and at molecular weights similar to those in mammary epithelial cells, as determined by immunoblotting with antibodies specific for each kinase (data not shown but see below).

Etoposide-induced activation of JNKs in the parotid C5 and submandibular C6 cell lines was examined using the GST-jun kinase assay.[21] The activation of JNK in etoposide-treated parotid C5 cells could occasionally be detected by 2 h, and was readily detectable by 4 h. Maximal activation of JNK was detected at 8–10 h post treatment in the parotid C5 cells (Figure 3). We were able to detect only a minimal level of JNK activity in etoposide-treated submandibular C6 cells (Figure 3, lanes 9–16). In agreement with data from the parotid cells, this minimal level of JNK activity could be shown to peak at 8–10 h by overexposure of the film (data not shown). JNK activation in the submandibular C6, as well as in the parotid C5 cells, could be activated by anisomycin (Figure 3, lane 17).

A dose-response curve was established with the parotid C5 cell line (Figure 4A). Cells were stimulated with 0–200 μM etoposide for 8 h. Maximal activation of JNK activity was detected at approximately 10 μM etoposide (Figure 4A). The ability of the same concentrations of etoposide to induce the release of histone-associated low molecular weight DNA from the nucleus was examined with the Cell Death Detection Kit (Figure 4B). Concentrations as low as 5 μM were effective; near maximal effects were apparent at 10 μM etoposide (Figure 4B).

Etoposide decreases the amount of activated ERK1 and ERK2, but does not activate p38/*HOG1*

The difference in the effect of etoposide upon JNK activation between the parotid C5 and submandibular C6 cells described in Figure 3 above suggested that we should also

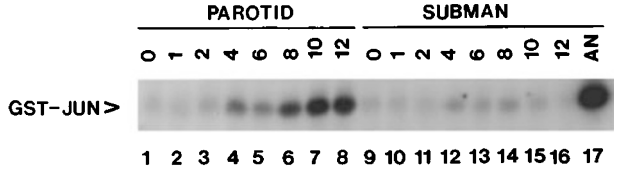

Figure 3 Etoposide induced activation of JNK in parotid C5 cells. The parotid C5 (lanes 1–8) and submandibular C6 (lanes 9–17) cell lines were treated with 100 μM etoposide for 0–12 h. At the indicated times cell lysates were prepared and assayed for JNK activity using the GST-jun kinase assay.[21] Cells were also treated with 20 μg anisomycin for 30 min as a positive control.[8] The reaction products were displayed on a 10% SDS polyacrylamide gel. An autoradiogram of the dried gel is shown. Time of stimulation in hours at the top of each lane and the lane number is indicated at the bottom. The position of the GST-jun fusion protein is indicated by the arrowhead on the left side of the panel. The results shown are representative of six independent studies conducted with both cell lines

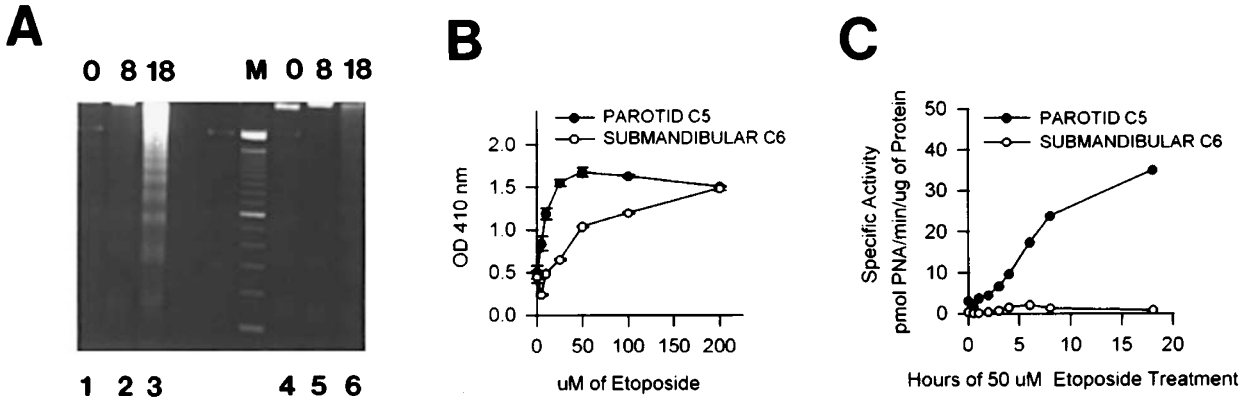

Figure 2 Etoposide induces apoptosis in the parotid C5 cell line but not in the submandibular C6 cell line as determined by a variety of criteria. (**A**) Subconfluent monolayers of the parotid C5 (lanes 1–3) and submandibular C6 (lanes 4–6) cells were treated with 20 μM etoposide for 8 or 18 h. DNA was isolated as described in the Materials and Methods from control or etoposide-treated cells. The isolated DNA was analyzed on a 1.5% agarose gel to examine the extent of DNA fragmentation induced in either cell type. Control cells are shown in lanes 1 and 4, cells treated with etoposide for 8 h in lanes 2 and 5, and cells treated with etoposide for 18 h are shown in lanes 3 and 6. The results shown here are representative of three independent studies with both cell lines. (**B**) Subconfluent monolayers of the parotid C5 and submandibular C6 cells were treated with 0–200 μM etoposide for 18 h. The cells were harvested and the amount of histone-associated low molecular weight DNA present in the cytoplasm of the cells was determined using the Cell Death Detection Assay Kit (Boehringer Mannheim). Treated parotid C5 cells are shown in the curve with the closed circles, while treated submandibular C6 cells are shown in the curve with the open circle s. The data shown is representative of three studies. (**C**) Subconfluent monolayers of the parotid C5 and submandibular C6 cells were treated with 50 μM etoposide for 0–18 h. At the indicated times, the cells were assayed for the presence of activated caspase-3 using the Caspase-3 Cellular Activity Assay Kit PLUS from BIOMOL. The time of treatment with 50 μM etoposide is shown on the ordinate and the specific activity of caspase-3 in released pNA min^{-1} μg^{-1} of protein is indicated on the abscissa. The indicated numbers are the average of four replicate samples

examine the effect of 100 μM etoposide upon both the ERK and p38/*HOG1* families of MAP kinases. We chose to examine the activation of ERK1, ERK2, and p38 using antibodies that recognize the dually phosphorylated, active forms of ERK2 and p38. The anti-ACTIVE ERK2 antibody also shows some crossreaction with ERK1, allowing examination of both ERK1 and ERK2 on the same gel.

Subconfluent monolayers of both the parotid C5 and submandibular C6 cells were treated with 100 μM etoposide. Activated ERK1 and ERK2 could be detected in both the untreated parotid C5 and submandibular C6 cells (Figure 5A). Stimulation of the parotid C5 cells with 100 μM etoposide resulted in a decrease in the amount of activated ERK1 and ERK2 which was first detectable after 4 h treatment with etoposide (Figure 5A). By 10–12 h activated ERK1 and ERK2 were difficult to detect (Figure 5A). There was a very small but detectable decrease in the amount of activated ERK2 in etoposide-treated submandibular C6 cells (Figure 5A). Uniform loading of the gels was demonstrated by reprobing the blots with an anti-ERK antibody that recognizes all ERK1 and ERK2 protein regardless of its phosphorylation state (Figure 5B).

In contrast with the results obtained with ERK1 and ERK2, we did not detect any effect of etoposide upon the activation of p38 (Figure 5C). Activated p38 was readily detectable in lysates of anisomysin-treated cells, demonstrating the p38 could be activated in these cells (Figure 5C, lanes 9 and 18). Both cell lines expressed p38 and there was no change in the levels of p38 in either cell line following etoposide treatment (Figure 5D).

The extent of JNK activation and ERK inactivation shown in Figures 3 and 5 was quantitated by densitometry and the kinetics of activation/inactivation compared in Figure 6. The amount of activated ERK1 and ERK2 decreases with the same kinetics following etoposide-treatment (Figure 6). Clearly the decrease in ERK

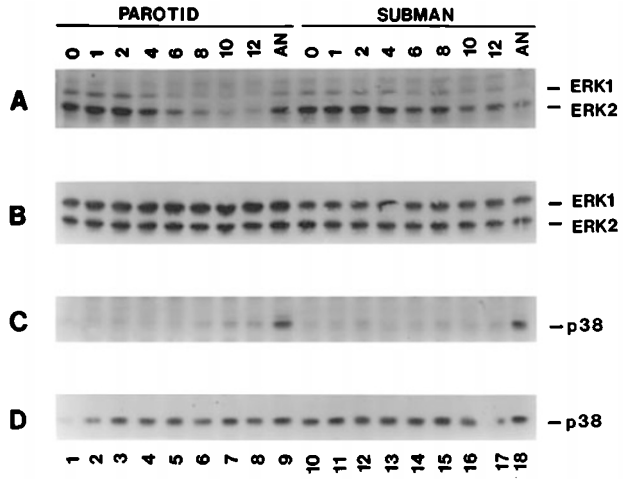

Figure 5 Does etoposide treatment result in activation of ERK1/2 or p38? The parotid C5 and submandibular C6 cell lines were treated with 100 μM etoposide for 0–12 h, then whole cell lysates prepared for immunoblot analysis with antibodies that recognize the activated ERK2 and ERK1, or activate p38. In (**A**) the parotid C5 (lanes 1–9) and the submandibular C6 (lanes 10–18) cells were treated with 100 μM etoposide for 0–12 h and whole cell lysates prepared. 25 μg amount of each whole cell lysate was resolved on an 8% polyacrylamide gel and immunoblotted with anti-ACTIVE ERK2 which crossreacts with both ERK1 and ERK2. In (**B**) the immunoblot shown in (**A**) was stripped and reprobed with an anti-ERK antibody that recognized both ERK1 and ERK2. The positions of both ERK1 and ERK2 are noted on the right side of each panel. In (**C**) the parotid C5 (lanes 1–9) and the submandibular C6 (lanes 10–18) cells were treated with 100 μM etoposide for 0–12 h, or with 20 μg ml^{-1} anisomycin for 60 min. Whole cell lysates were prepared and 25 μg total cellular protein resolved on 8% polyacrylamide gel. The immunoblot was probed with an anti-ACTIVE p38 antibody. In (**D**) the immunoblot shown in (**C**) was stripped and reprobed with an anti-p38 antibody to demonstrate equal loading. The position of p38 in (**C**) and (**D**) is indicated by the arrowhead on the right side of the panel. The time of stimulation in hours with etoposide is indicated by the numbers at the top of each lane. Anisomysin controls are indicated by AN. Lane numbers are indicated at the bottom

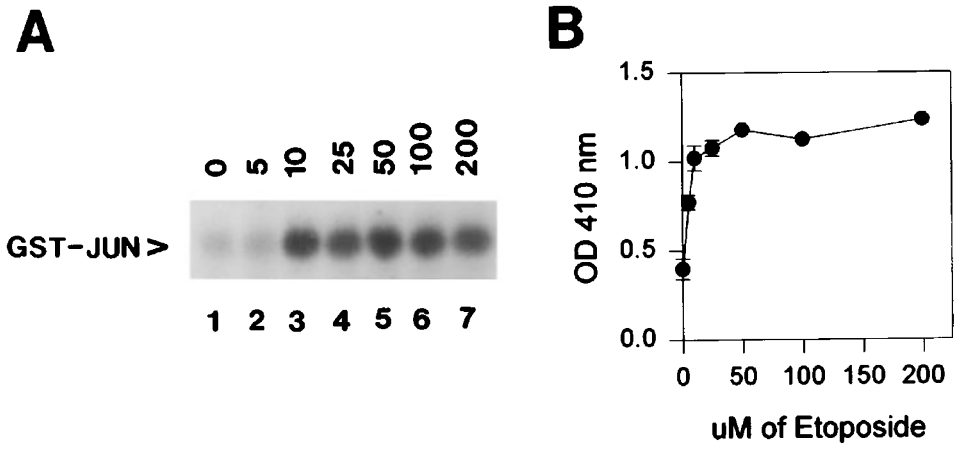

Figure 4 Dose response of JNK activation in response to etoposide. Duplicate plates of the parotid C5 cells were stimulated with 0–200 μM etoposide for 8 h. In (**A**) one plate of treated cells was lysed in JNK lysis buffer and the cell lysates used in the GST-jun kinase assay as described above. The concentration of etoposide is indicated at the top of each lane and the position of GST-jun is indicated in the left side of the panel. In (**B**), the duplicate plate was lysed as described in the instructions for the Boehringer Mannheim Cell Death Detection Assay and the samples processed as recommended for use in the anti-histone ELISA. The concentrations of etoposide used to treat the parotid C5 cells is plotted *versus* the increase in the OD_{410} of the anti-histone ELISA. All points represent the average of triplicate samples and standard error is indicated by the error bars

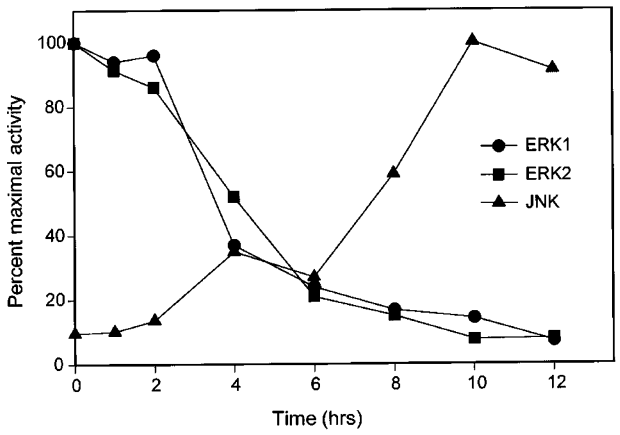

Figure 6 Comparison of ERK inactivation with JNK activation in etoposide-treated parotid C5 cells. The intensity of the bands corresponding to phosphorylated GST-jun and activated ERK1 and ERK2, presented in Figures 3 and 5A, respectively, were quantitated by densitometry. The amount of activated ERK1 and ERK2 present in untreated cells was set as 100%. The maximal amount of phosphorylated GST-jun was detected following 10 h treatment with etoposide, and this was taken as 100%. The per cent of activated ERK1 (●), ERK2 (■), and GST-Jun kinase activity (▲) are plotted *versus* the time of treatment (in hours) with 50 μM etoposide

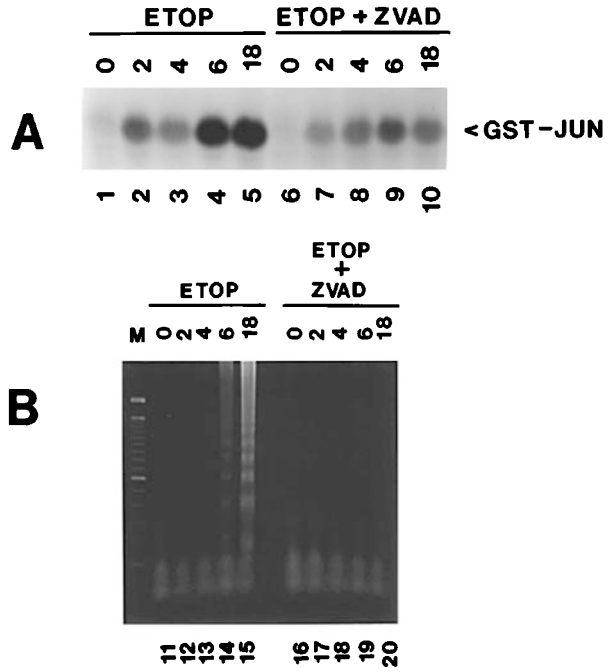

Figure 7 ZVAD inhibits etoposide activation of JNK. In (**A**) parotid C5 cells were either left untreated (lanes 1–5), or pretreated with 50 μM ZVAD (lanes 6–10) prior to treatment with 50 μM etoposide for 0–18 h. At the indicated times, the cells were lysed and processed for use in the GST-jun kinase assay as described in Figure 4 above. The time of treatment in hours is shown at the top of each lane. Lane numbers are indicated at the bottom of each lane, and the position of GST-JUN is indicated on the right side of the panel. In (**B**) parotid C5 cells were treated in an identical manner, however, the cells were processed for analysis by the DNA ladder technique. Cells were either left untreated (lanes 11–15), or pretreated with 50 μM ZVAD (lanes 16–20) prior to treatment with 50 μM etoposide for 0–18 h. At the indicated times, the cells were lysed and processed to detect DNA fragmentation as indicated by the presence of a DNA ladder. The time of treatment in hours is shown at the top of each lane, and lane numbers are indicated at the bottom of each lane. The lane marked 'M' contains DNA size markers

activation precedes the large increase in JNK activation, although the relationship between these events is not clear. Presumably the inactivation of ERKs occurs through the action of MAP kinase phosphatases since there is no decrease in the amount of either ERK1 or ERK2. JNK activation is likely to be regulated by upstream JNK kinases and should proceed in a manner independent of ERK inactivation.

ZVAD inhibits etoposide-induced activation of JNK

Activation of caspases is a critical event in apoptosis.[22] Caspase activation is also required for the activation of protein kinases that stimulate apoptosis, and activation of these kinases leads to further increases in caspase activation.[23–25] JNK and p38 activation lie downstream of caspase activation.[25,26] We were therefore interested in determining whether caspase activation was required for activation of JNK.

Parotid C5 cells were treated with 50 μM ZVAD for 30 min prior to the addition of 50 μM etoposide. Activation of JNK, inactivation of ERKs, and induction of DNA fragmentation was examined at 0–18 h after the addition of etoposide (Figure 7). Consistent with the results shown in Figure 3, etoposide-induced activation of JNK as determined by the GST-jun kinase assay (Figure 7A, lanes 1–5). Although a low level of JNK activation could be detected at 2–4 h in both the untreated and ZVAD-treated parotid C5 cells (compare lanes 1–3 with 6–8 in Figure 7), the increase that was observed at 6–18 h following etoposide treatment was severely dampened in the parotid C5 cells treated with 50 μM ZVAD (Figure 7A, compare lanes 4 and 5 with lanes 9 and 10). As a control, we examined the ability of ZVAD to block etoposide-induced DNA fragmentation (Figure 7B). Fifty μM ZVAD completely blocked the appearance of a DNA ladder (Figure 7B, lanes 11–15 *versus* 16–20). Morphological observation also confirmed the ability of 50 μM ZVAD to block etoposide-induced apoptosis (data not shown). These data suggest that etoposide induced JNK activation in salivary cells may require caspase activation or activity.

Discussion

In this study we provide evidence that two recently established salivary gland acinar cell lines[27,28] differ in their sensitivities to a chemotherapeutic drug which can kill cells by inducing apoptosis. These represent the only established salivary acinar cell lines. Several criteria indicate that etoposide induces apoptosis in the parotid C5 cell line, but not the submandibular C6 cell line: (1) induction of cytoplasmic blebbing and nuclear condensation; (2) induction of a DNA ladder indicating fragmentation of nuclear DNA; (3) appearance of histone-associated low molecular weight DNA in the cytoplasm of etoposide-treated parotid C5 cells; (4) cleavage of poly(ADP-ribose) polymerase (PARP) (data not shown), and (5) activation of caspase-3. Although

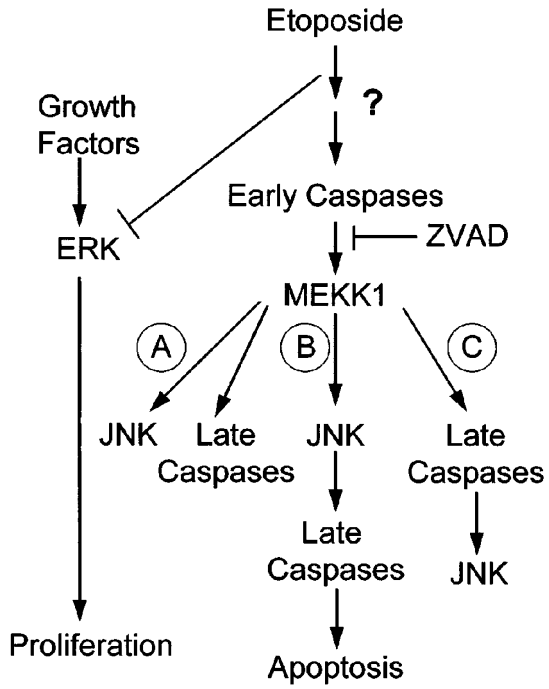

Figure 8 Model for the role of JNK in salivary gland apoptosis. See the text for discussion. Three different models could explain the etoposide-induced activation of JNKs, and the ability of ZVAD to block JNK activation and apoptosis. In (**A**) the activation of both JNK and late caspases occur independently from each other, however, they both lie downstream of MEKK1 which is cleaved and activated by early caspases. In (**B**), activation of JNKs is regulated by MEKK1, and the activation of late caspases is dependent upon JNK activation. In (**C**), the cleavage of MEKK1 by early caspases leads to the activation of the late caspases which in turn activate JNK. In each model the effect of ZVAD is to prevent cleavage of MEKK1 which results in its catalytic activation

cytoplasmic blebbing was observed in a small number of etoposide-treated submandibular C6 cells, and a low level of DNA fragmentation could be detected in the histone release assay, most of these cells did not undergo apoptosis. Although both cells lines were derived from cultures of salivary gland acinar cells transfected with a SV40 T antigen encoding plasmid, only the parotid C5 cell line expresses SV40 T antigen.[27,28] Preliminary data suggests however, that etoposide induces apoptosis in primary parotid cells, but not submandibular cells. This suggests that there may be an inherent difference in the sensitivity of these cells to etoposide that cannot be explained by the presence of SV40 T antigen, or the levels of p53 or RB (unpublished data, L.M. Deisher, K.A. Barzen, and D.O. Quissell).

Treatment of the parotid C5 cell line, but not the submandibular C6 cell line, with etoposide resulted in the activation of JNKs and in a decrease in the level of activated ERK. The time course and dose response curves for both events were similar. Furthermore, the dose response curves for JNK activation, ERK inhibition, and induction of DNA fragmentation as measured by the release of histone-associated low molecular weight DNA fragments into the cytoplasm, were comparable. This suggests that the activation of JNK coupled with a decrease in the level of activated ERK in these cells could lead to the induction of apoptosis in the parotid C5 cells.

ERK1 and ERK2 have been demonstrated to be critical in mitogenic signaling and oncogenesis.[29,30] There is also evidence that activation of ERKs stimulates protection against factors that mediate apoptosis.[31,32] In contrast to data on ERKs, there is evidence that JNK activation is critical and sometimes sufficient to induce apoptosis. JNKs are required for apoptosis induced by nerve growth factor (NGF) withdrawal in PC12 cells,[32] and sympathetic neurons.[33] Furthermore, JNK activation is required for anoikis of MDCK cells.[34] Dominant negative mutants of JNKs can block apoptosis of HEK 293 cells induced by gamma irradiation and UVC,[35] and UV-induced apoptosis of small cell lung carcinoma cells.[36] Furthermore, dominant negative mutants of JNK also blocked Fas-mediated activation of JNK and apoptosis in SHEP neuroblastoma cells.[37] Treatment of U937 cells with antisense oligonucleotides that inhibit JNK expression suppressed caspase activation following treatment with etoposide or camptothecin,[38] implicating JNK activity in the activation of caspases required for apoptosis. JNK activation has also been demonstrated to antagonize the anti-apoptotic effect of bcl-2.[39] In contrast to studies demonstrating that JNK activation may induce apoptosis, the expression of a dominant negative mutant of c-*jun* did not affect apoptosis induced by ionizing irradiation,[40] or cisplatin.[41] Other studies have also indicated that JNK activation can be disassociated from apoptosis.[42,43]

The activation of caspases has been shown to be critical in the induction and execution of apoptosis (for reviews see[22]). Numerous cellular proteins are cleaved by caspases. The cleavage of various proteins has been observed to occur in three different groups: an early group, an intermediate group, and a late group, suggesting an ordered sequence of proteolytic events leading to irreversible death.[44] The activation of two different protein kinases, MEKK1[23] and Mst1,[24] requires caspase activation, and activation of these kinases leads to further activation of caspases. Thus at least two different protein kinases whose activation may be important in caspase activation appear to be activated in a caspase-dependent manner. Other investigators have shown that caspase activation is required for activation of JNKs and p38.[25,26]

Treatment of the parotid C5 cells with 50 μM ZVAD resulted in an inhibition of the increase in JNK activity that occurs at 6–18 h after treatment with etoposide. This suggests that caspases are required for activation of JNK. There was no effect of ZVAD upon the inactivation of ERK1, and ERK2. Etoposide induces caspase-dependent cleavage of MEKK1, which leads to its catalytic activation.[23] This observation suggests that JNK activation may be mediated by MEKK1. The suppression of ERK activation suggests that JNK activation may be mediated by MEKK1. The suppression of ERK activation suggests that MEKK1 does not function to activate ERKs, and the lack of an effect of ZVAD upon ERK inactivation, suggest that the latter event occurs in a caspase-independent manner. There are three different models that can explain the relationship between JNK activation and the activation of

'late' caspases (Figure 8). As shown in Figure 8A, JNK activation could occur independently of late caspases. Alternatively, JNK activation could be required for the activation of 'late' caspases (Figure 8B), or the late caspases could be required for the activation of JNK (Figure 8C). Although ZVAD could act at several different points in these pathways, the most logical point for it to function is to suppress the cleavage and activation of MEKK1. Alternatively, ZVAD could inhibit events that lie downstream of MEKK1 cleavage, however, this would not be consistent with the results of Widman *et al*.[23] The results of Graves *et al*.[24] also suggest that Mst1 could function as an activator of JNK in a caspase-dependent manner.

It is not clear whether the decrease in the level of activated ERK is a direct consequence of etoposide treatment, or whether it occurs as a result of JNK activation. The fact that ERK inactivation occurs in cells treated with both ZVAD and etoposide suggests that the inactivation of ERK is not necessary and sufficient to induce apoptosis. Future studies will utilize constitutively activated and dominant negative mutants of these different molecules to provide insights into the mechanisms regulating apoptosis in salivary gland acinar cells. As noted above, these insights may also be relevant to the development of therapeutic approaches that could be used to prevent inadvertent damage to the salivary gland in patients undergoing chemotherapy or head and neck irradiation.

Materials and Methods

Cells and cell culture

The isolation of immortalized salivary parotid C5 and submandibular C6 cell lines has been described elsewhere.[27,28] Cells were cultured on Primaria™ 60 mm culture dishes (Falcon Plastics, Franklin Lakes, NJ, USA). The culture media used was DMEM/F12 (1 : 1 mixture) supplemented with 2.5% fetal calf serum, 5 μg ml^{-1} transferrin, 1.1 μM hydrocortisone, 0.1 μM retinoic acid, 2.0 nM T3, 5 μg ml^{-1} insulin, 80 ng ml^{-1} epidermal growth factor, 4 mM L-glutamine, 50 μg ml^{-1} gentamicin sulfate, and a trace element mixture. Epidermal growth factor was from Collaborative Biomedical Products (Bedford, MA, USA) and the trace element mixture was purchased from Biofluids (Rockville, MD, USA). All other tissue culture reagents were obtained from GIBCO/BRL (Gaithersberg, MD, USA). Cell cultures were transferred twice a week while subconfluent, since both cell lines undergo density-dependent apoptosis when they become confluent.[27,28]

Kinase assay for JNK activity

Cells to be analyzed were stimulated with varying (0–200 μM) concentrations of etoposide in tissue culture media for 0–18 h. Cells were lysed on the plate in JNK lysis buffer [25 mM HEPES, pH 7.7, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.1% Triton X-100, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 4 μg ml^{-1} Aprotinin (Calbiochem, La Jolla, CA, USA)], the cells removed with a rubber policeman, and the lysate was allowed to sit on ice for 30 min. The lysates were clarified by spinning at 12 500 r.p.m. for 5 min in a refrigerated Savant SRF13K microfuge. Protein concentrations of the clarified lysate were determined by the Pierce BCA protein assay (Rockford, IL, USA), and 300 μg total cellular protein was used in each assay. A 100 μl volume of a 10% suspension of GST-c-*jun* (1-79) was added to 300 μg total cellular protein in a final volume of 1 ml, and incubated for 2 h at 4°C. The beads were then washed three times with 20 mM HEPES, pH 7.7, 50 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.05% Triton X-100. Forty μl of 50 mM β-glycerophosphate, pH 7.6, 0.1 mM sodium orthovanadate, 10 mM $MgCl_2$, 20 μM ATP containing 10 mCi [γ-^{32}P]ATP (5000 c.p.m. pmol^{-1} in the final reaction) was added to the washed beads and the reaction was incubated at 30°C for 20 min. The reactions were terminated by the addition of 10 μl 5 × SDS sample buffer, boiled, and the reaction products resolved on a 10% SDS polyacrylamide gel. The position of GST-jun was determined by staining the gel, and the extent of GST-jun phosphorylation was determined by autoradiography. In some studies the cells were treated with 100 μM anisomycin (Calbiochem, La Jolla, CA, USA) as a positive control since ansiomycin has been shown to be a potent activator of JNKs.[8,45–47]

The GST-c-Jun (1-79) expression vector was kindly provided by Dr Lynn Heasley (University of Colorado Health Sciences Center, Denver, CO, USA), and the fusion proteins were prepared as described.[36] A 5 ml overnight culture of the bacteria was diluted to 500 ml and allowed to grow until an OD_{600} of 0.6. IPTG was added to a final concentration of 0.4 mM for 3 h, after which the bacteria were collected by centrifugation and the pellets frozen at −20°C. The pellets were thawed, resuspended in 10 ml NTEN [20 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA, 2 mM DTT, 0.5% NP-40, 100 U ml^{-1} Aprotinin (Calbiochem, La Jolla, CA, USA)], and 200 μl of 1 mg ml^{-1} lysozyme added. After 1 h on ice, the bacteria were sonicated for 2 min, the lysates clarified by centrifugation at 10 000 r.p.m. for 10 min, and the supernatant fluid transferred to a new tube. A 1.5 ml volume of a 50% slurry of glutathione agarose (Pharmacia Biotech, Piscataway, NJ, USA) was added, and the tube was placed on a rocking platform for 1 h at 4°C. The beads were washed three times with NTEN buffer, and resuspended in JNK lysis buffer to a final concentration of 10%.

Immunoblotting

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 2 mM EGTA, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM sodium orthovanadate) or JNK lysis buffer, and the lysates clarified by spinning at 10 000 r.p.m. in a Savant RCF13K refrigerated microfuge for 30 min. Protein concentration was determined as described above. A 25–50 μg amount of each cell lysate was resolved on an 8% gel, transferred to an Immobilon membrane, and immunoblotted with the desired antibody as described previously.[48,49]

Anti-JNK1, anti-JNK2, and anti-p38 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-ACTIVE JNK antibody and anti-ACTIVE p38 antibodies were obtained from New England Biolabs (Beverly, MA, USA). Anti-ACTIVE ERK2 and anti-ACTIVE JNK antibodies were obtained from Promega Biotechnology (Madison, WI, USA). An anti-MAP kinase antibody which cross-reacts with both ERK1 and ERK2, and an anti-PARP antibody were obtained from Upstate Biotechnology (Lake Placid, NY, USA).

Detection of DNA fragmentation

The fragmentation of cellular DNA was examined by the appearance of a DNA ladder. Cells to be examined were scrapped off the plate in tissue culture medium, pelleted, and washed twice with phosphate-buffered saline (PBS). After the second wash the cells were

resuspended in 2 ml PBS, transferred to a 2 ml microfuge tube, and the cells pelleted. The cells were resuspended in 2 ml 70% ethanol to fix the cells and stored at $-20^{\circ}C$ overnight. The cells were quickly pelleted and the ethanol removed. The cells were resuspended in 40 μl phosphate-citrate buffer (0.2 M Na_2HPO_4, 25 mM citric acid, pH 7.8), and incubated at room temperature for 30–60 min. The extract was clarified at 10 000 $\times g$ for 2 min, and the supernatant transferred to a new tube. The samples were concentrated in a Speed Vac, resuspended in 50 μl of 10 mM Tris, pH 7.4, 1 mM EDTA, and 3 μl 0.25% NP-40 added. A 6 μl aliquot of 0.5 mg ml^{-1} RNase was added, and the solution was incubated at 37°C for 30 min. A 3 μl aliquot of 20 mg ml^{-1} proteinase K was added, and the incubation continued for another 1 h at 37°C. Loading dye was added and 5 μg of DNA from each sample was analyzed on a 1.5% agarose gel.

Detection of histone-associated, cytoplasmic, low molecular weight DNA

DNA fragmentation was also assayed using a Cell Death Detection Assay kit from Boehringer Mannheim (Indianapolis, IN, USA). This assay detects the appearance of histone-associated low molecular weight DNA in the cytoplasm of cells and was performed in accordance with the manufacturer's recommendations.

Activation of caspase-3

The activation of caspase-3 was detected with the Caspase-3 Cellular Activity Assay Kit PLUS obtained from BIOMOL Research laboratories (Plymouth Meeting, PA, USA). The assays were conducted in accordance with the manufacturer's recommendations. ZVAD (Z-Val-Ala-Asp(O-methyl)-CH_2F) was obtained from Enzyme Systems (Livermore, CA, USA).

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