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Role of caspases in human renal proximal tubular epithelial cell apoptosis

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Abstract

In the present study, we have used an in vitro model of apoptosis using primary human renal proximal tubular epithelial (RPTE) cells to investigate the mechanisms involved in renal cell apoptosis. Treatment of RPTE cells with okadaic acid for 24–48 h induced apoptosis in a concentration-dependent manner. Apoptosis was accompanied by the activation of the p38 mitogen-activated protein kinase (MAPK) pathway followed by the activation of caspase-9, -3, and -7. The induction of caspase activity correlated with the proteolytic cleavage of β-catenin, suggesting that β-catenin is a caspase substrate. The caspase inhibitor, Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), resulted in a dose-dependent inhibition of apoptosis and β-catenin cleavage. These data suggest that okadaic acid-induced apoptosis is p38 MAPK and caspase-dependent and that proteolytic cleavage of β-catenin by caspases is likely to be a downstream molecular event associated with the morphological and cytoskeletal changes induced during apoptosis.

Keywords: Apoptosis; Caspase; β-Catenin; p38; Polycystic kidney disease; Okadaic acid

1. Introduction

Apoptosis is a genetically controlled, active form of cell death that plays an important role in tissue maintenance and organ homeostasis (Jacobson et al., 1997; Raff, 1998). Dysregulated apoptosis can be induced in response to pathological conditions as well as environmental insults. Excessive apoptotic cell death has been implicated in the pathogenesis of a number of human diseases including polycystic kidney disease. Kidneys of patients with polycystic kidney disease have abnormally high levels of apoptotic cells present in cystic tubules, cyst walls and glomeruli and it has been proposed that apoptosis contributes to the progressive loss of renal tissue in this disease (Woo, 1995; Lanoix et al., 1996). Furthermore, dysregulated apoptosis has been reported in the interstitium of kidneys from an animal model for polycystic kidney disease, the cpk mouse (Ali et al., 2000), which supports the suggestion that interstitial accumulation of mononuclear cells and subsequent scarring contributes to the loss of renal function seen in some patients with polycystic kidney disease (Grantham, 1995).

Caspases, a family of evolutionarily conserved cysteine proteases, play an important role in apoptotic cell-death (Alnemri, 1997; Nuñez et al., 1998; Kidd, 1998; Thornberry and Labzebnik, 1998; Earnshaw et al., 1999). To date, 14 caspases have been identified and have been classified into three subfamilies (Hu et al., 1998). They are normally synthesized as proenzymes that are proteolytically processed to their active forms upon induction of apoptosis. The activated caspases selectively cleave specific intracellular substrates, including a variety of regulatory and structural proteins and enzymes. Although the importance of the caspase family in apoptosis has been established, the signaling pathways mediating apoptotic cell death and the role of different members of the caspase family are less well defined. To date evidence suggests that caspases-3, -7 and -9 are key members of the caspase cascade (Budihardjo et al., 1999) and, as such, were the caspases evaluated in the present study.

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Recent studies have revealed that the p38 mitogen-activated protein kinase (MAPK), a member of the serine/threonine kinases family that is activated by dual phosphorylation on a Thr-Gly-Tyr motif and mediates intracellular signal transduction, is involved in apoptosis (Ono and Han, 2000). In the present study, we have, therefore, evaluated the mechanisms involved in renal proximal tubule epithelial (RPTE) cells using a phosphotase inhibitor, okadaic acid, which has been shown to induce apoptosis via p38 MAPK-dependent (Davis et al., 1996) and caspase-dependent (Jensen et al., 1999) pathways. In addition, we evaluated the effect of caspase inhibition on β-catenin cleavage since there is evidence that β-catenin is a substrate for caspases (Brancolini et al., 1997). Our data indicate that okadaic acid-induced apoptosis in the renal proximal tubular epithelial cells involves the activation of a caspase cascade that lies downstream of p38 kinase activation and that blocking the caspase cascade protects cells from apoptosis.

2. Materials and methods

2.1. Induction and measurement of apoptosis

Human RPTE cells obtained from Clonetics (San Diego, CA) were grown in complete growth medium to about 70% confluence in six-well plates in a 37 °C, 5% humidified incubator. Cells were transferred to basic medium containing 0.5% fetal bovine serum overnight. Cytokine-fasted cells were cultured for periods of 6, 12, 24, 36, and 48 h with 10, 30, 50 and 100 nM okadaic acid (Calbiochem, La Jolla, CA), or 500 nM okadaic acid for periods of 2, 4, 6, 8, 9, 10, 12 and 24 h to induce apoptosis. To identify the dose of caspase inhibitor that inhibited cell death, cells were cultured with 5, 10, 25, 50 and 100 μM Z-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) (Enzyme Systems Products, Livermore, CA) for 1 h prior to the addition of okadaic acid (100 or 500 nM).

Apoptotic cell death was determined by either DNA fragmentation or cellular ADP/ATP ratios. DNA fragmentation was determined using DNA laddering and the Cell Death Enzyme Linked Immunosorbent Assay (ELISA) Plus kit (Boehringer Mannheim, Indianapolis, IN). For cell death ELISA determination, cells were lysed and assayed according to the manufacturer's protocol. ADP/ATP levels were measured using the ApoGlow kit (Lumitech, Nottingham, UK).

2.2. Measurement of caspase activity

The 70–80% confluent, overnight, cytokine-fasted cells in T150 flasks were treated with okadaic acid, as described above. The adherent and floating cells were collected and washed three times with cold phosphate buffered saline (PBS). The washed cells were pelleted and resuspended in buffer (25 mM HEPES, pH 7.5, 10% sucrose, 0.1% 3-([3-Cholamidopropyl]dimethylammonio)-1-propanesulfonate[-CHAPS], 2 mM dithiothreitol, 5 mM EDTA, 1 mM phenyl methyl sulfono fluoride, and 1 μM pepstain A), and allowed to swell for 20 min on ice. The suspension was passed through a 25-gauge syringe needle 12 times, and the homogenate was centrifuged at 4 °C for 1 h at 100,000 × *g*. Protein concentrations were determined by the Bradford assay from Bio-Rad (Hercules, CA). The resulting supernatants were stored at − 70 °C until used for assays.

Caspase activity was measured in cell extracts by comparing activity profiles generated using caspase-selective substrates and recombinant caspases. The substrates were either obtained commercially or prepared in-house. Peptide-7-amino-4-methyl-courmarin (AMC) cleavage assays were conducted in 96-well microtitre plates in a total volume of 100 μl. Assay buffer for Ac-Tyr-Val-Ala-Asp (YVAD)-AMC contained 25 mM K HEPES at pH 7.5, 10% sucrose, 0.1% CHAPS, 1 mM dithiothreitol; assay buffer for Ac-DEVD-AMC and Ac-Asp-Glu-Met-Asp (DQMD)-AMC contained 25 mM K HEPES at pH 7.5, 0.1% CHAPS, 50 mM KCl, 1 mM dithiothreitol; assay buffer for Ac-LEED-AMC contained 25 mM KOAc at pH 5.8, 1 mM EDTA, 10% sucrose, 0.1% CHAPS; and assay buffer for Ac-IETD-AMC contained 75 mM Na 3-morpholinopropanesulfonic acid (MOPS) at pH 7.5, 10% glycerol, 0.25 mM EDTA, and 1 mM dithiothreitol. Extracts containing 5–20 μg of protein were diluted into assay buffer and preincubated for 10 min at 30 °C. Substrates were then added to a final concentration of 100 μM to initiate the reaction. Substrate cleavage was measured with a Cytofluor 4000 fluorescent plate reader (PerSeptive Biosystems) as an increase in fluorescence at an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

2.3. Western blot analysis

Extracts from RPTE cells treated with 100 nM for 24 h or 500 nM okadaic acid for different period of times were subjected to 4–12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. Western blot analysis was performed with the specific antibodies: anti-β-catenin, anti-α-catenin from Transduction Laboratories (Lexington, KY), anti-GAPDH from Research Diagnostics (Minneapolis, MN), caspase-3, -7, -9 and phospho-p38 antibodies from New England Biolabs (Beverly, MA). The immunogens of monoclonal antibodies anti-β-catenin and anti-α-catenin are 210 and 177 amino acids located in the carboxyl terminal region of the proteins, respectively. The bands were visualized by Super Signal chemiluminescent substrate (Pierce Laboratories, Rockford, IL).

2.4. Cell viability assay

Cell viability was quantitated by measuring the released cytoplasmic enzyme activity of lactate dehydrogenase (LDH) according to the manufacturer's instructions (Promega, Madison, WI). LDH which is a stable cytoplasmic enzyme present in all cells is rapidly released into the cell culture media

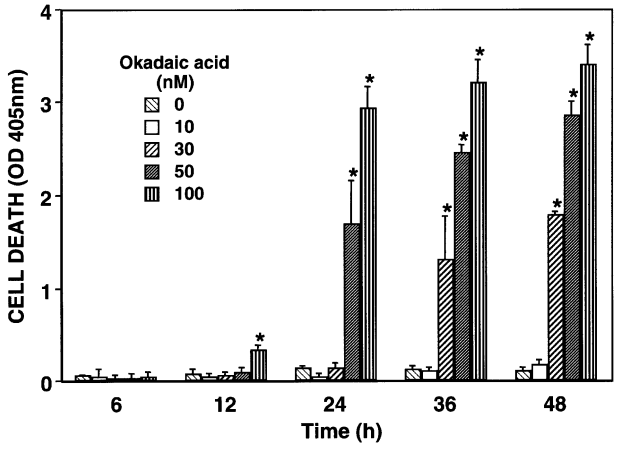

Fig. 1. Okadaic acid-induced apoptosis of RPTE cells as determined by cell death ELISA for cytoplasmic histone-associated-DNA fragments. $n=4$. * $P<0.05$ versus 0.

when the plasma membrane is damaged. LDH release was expressed as % release, which was calculated from the amount of LDH in the supernatants of experimental samples/maximum LDH present in the population. Maximum LDH is the amount of LDH available for release from an equivalent population of untreated cells.

2.5. Data analysis

Data are presented as mean ± S.E. and were analyzed statistically using an analysis of variance (SuperANOVA).

3. Results

Okadaic acid treatment of RPTE cells resulted in apoptosis as determined by a cell-death ELISA for fragmented DNA (Fig. 1). Okadaic acid-induced apoptosis was both concentration and time-dependent. Okadaic acid treatment also resulted in time-dependent activation of several caspases (Fig. 2). Western blot analysis indicated that caspase-9 processing occurred after 6 h of treatment, whereas caspase-3 and caspase-7 processing were observed after 8 h, indicating that the activation of caspase-9 is upstream of caspases-3 and -7. The reason for the apparent increase in total caspase-3 staining after OA treatment is unclear given that total caspase-7 and -9 staining was unaltered.

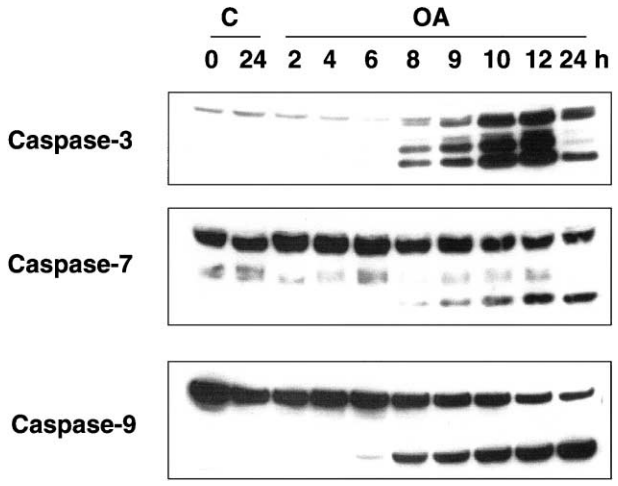

Fig. 2. Time course of okadaic acid-induced (OA) activation of caspase-3, -7 and -9 in RPTE cells as determined by Western blot analysis.

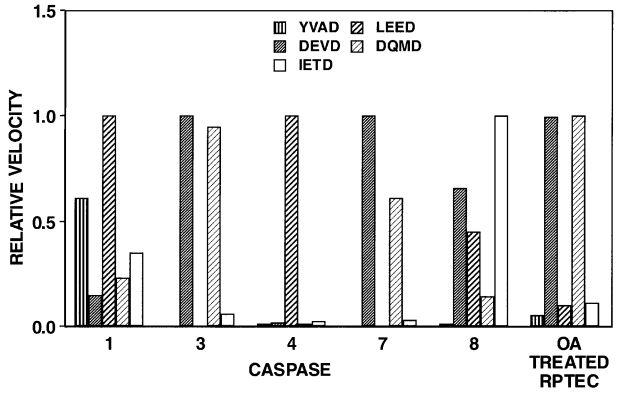

Fig. 3. Substrate profiles of human recombinant caspases and RPTE cell extracts treated with 100 nM okadaic acid (OA) for 24 h.

Activity of the processed caspases was confirmed by the evaluation of proteolytic activity. When the substrate specificity of various recombinant caspases and the 100 nM okadaic acid-treated for 24 h RPTE cell extracts were compared, the activity demonstrated a profile most similar to caspases-3 and -7 (Fig. 3). In addition, the okadaic acid-induced caspase activity was effectively inhibited by the caspase inhibitor, Z-VAD-fmk. 100 nM okadaic acid treatment for 24 h caused a 400-fold increase in DEVD cleavage

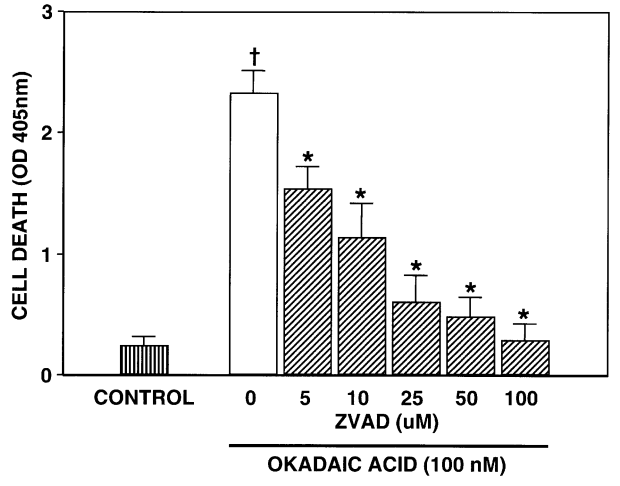

Fig. 4. Inhibition of okadaic acid-induced RPTE cell apoptosis by the caspase inhibitor Z-VAD-fmk at 24 h using an ELISA assay for cytoplasmic histone-associated-DNA fragments. $n=4-6$ observations. † $P<0.05$ versus control; * $P<0.05$ versus 0.

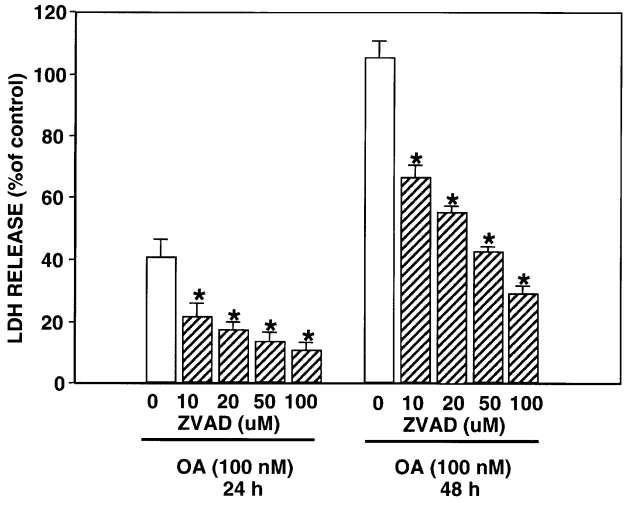

Fig. 5. Inhibition of okadaic acid-induced (OA) RPTE cell death at 24 and 48 h by the caspase inhibitor Z-VAD-fmk as determined by LDH release. Data are expressed as % LDH release relative to the total amount of LDH available for release determined from an equivalent population of untreated cells. $n=6$. * $P<0.05$ versus 0.

activity in RPTE cells as compared to untreated cells, and Z-VAD-fmk completely inhibited the DEVD cleavage activity.

Blocking the caspase activity by Z-VAD-fmk treatment resulted in the protection of the cells from okadaic acid-induced apoptotic cell death (Fig. 4). Quantitative assessment of cell viability by LDH leakage demonstrated that about 40% of cells underwent apoptotic cell death 24 h following 100 nM okadaic acid treatment (Fig. 5). Treatment with 100 μM Z-VAD-fmk demonstrated an almost complete inhibition of okadaic acid-induced cell death after 24 h. The protective effect could be extended to 48 h of

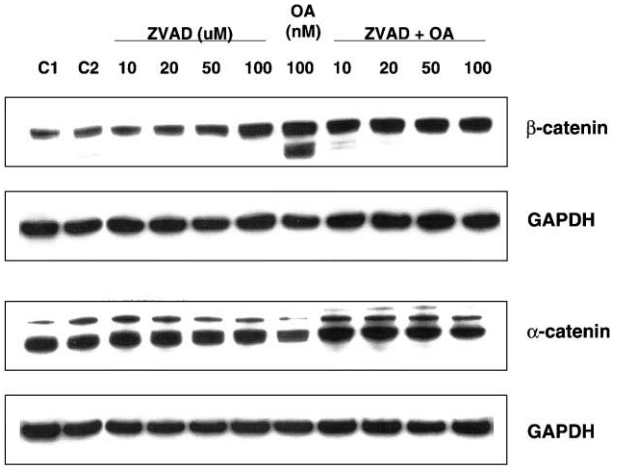

Fig. 6. Inhibition of okadaic acid-induced (OA) cleavage of β-catenin by the caspase inhibitor Z-VAD-fmk in RPTE cells, as determined by Western blot analysis (A). The lack of proteolytic cleavage of α-catenin was demonstrated using the same extracts (B). C1 = untreated cells, C2 = vehicle (1% DMSO)-treated cells. Equal loading was determined by reprobing with an anti-GAPDH antibody.

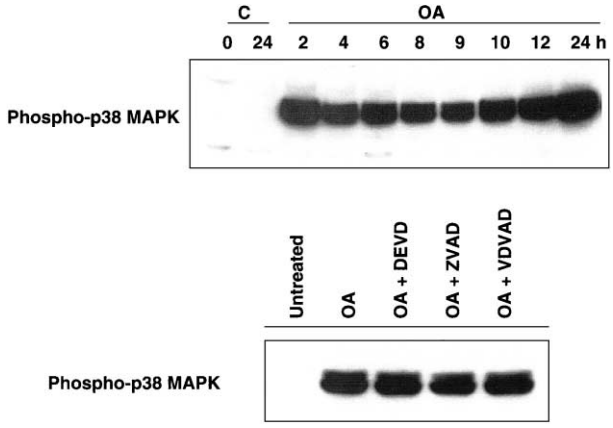

Fig. 7. Time course of okadaic acid (OA) (500 nM) induced p38 MAPK activation in RPTE cells as determined by Western blot analysis (top panel) and effect of caspase inhibition following 2 h of treatment (bottom panel).

treatment since the percentage of LDH release was about 70% lower as compared to the okadaic acid treatment alone.

Okadaic acid treatment of RPTE cells also resulted in the proteolytic cleavage of β-catenin, a multifunctional protein involved in cell adhesion and the Wnt signaling pathway (Fig. 6). The full length 92-kDa β-catenin was cleaved near the NH_2-terminus to produce a doublet of $\sim 62-70$ kDa. The proteolytic cleavage of β-catenin was completely inhibited by the addition of caspase inhibitor Z-VAD-fmk, suggesting the increased caspase activity 3/7 was responsible. In addition, most of the cleaved β-catenin was found in the floating population of cells (data not shown). There was no proteolytic cleavage of α-catenin under the same experimental conditions (Fig. 6). β-Catenin cleavage following okadaic acid induced-apoptosis also occurred in the presence of the proteosome inhibitor, lactacystin, indicating that it was not processed by the ubiquitin/proteosome pathway (data not shown).

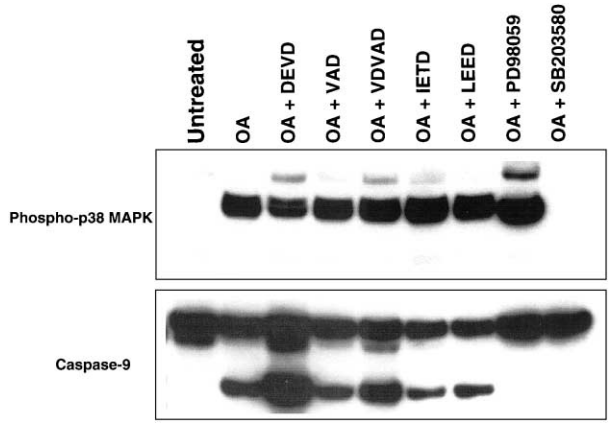

Fig. 8. Lack of inhibition of okadaic acid-induced (OA) p38 MAPK activation by caspase inhibitors in RPTE cells as determined by Western blot analysis (top panel). Inhibition of caspase 9 activation by the MAPK inhibitors PD98059 and SB203580 (bottom panel).

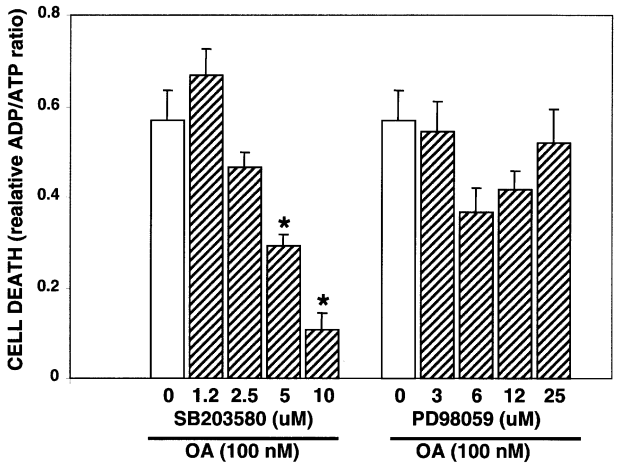

Fig. 9. Effect of the p38 inhibitor, SB203580, and the MEK inhibitor, PD98059, on okadaic acid-induced apoptosis of RPTE cells. $*P<0.05$ versus 0.

Okadaic acid-induced apoptosis was accompanied by the activation of p38 kinase determined using a specific phospho-p38 MAPK antibody. Okadaic acid-induced phosphorylation of p38 MAPK was observed 2 h following okadaic acid treatment and it was not affected by the caspase inhibitors Z-VAD-fmk, Z-DEVD-fmk or Z-VDVAD-fmk (Fig. 7). Although caspase-9 processing was not inhibited by Z-VAD-fmk, Z-DEVD-fmk, Z-VDVAD-fmk, Z-LEED-fmk and Z-IETD-fmk, caspase-9 activation was abolished when cells were treated with the MAPK inhibitors, SB203580 and PD98059 (Fig. 8). Furthermore, SB203580 but not PD98059 inhibited OA-induced apoptosis in RPTE cells (Fig. 9).

4. Discussion

There is growing interest in the possible role of apoptosis in renal disease, and in particular polycystic kidney disease, where evidence for apoptosis has been observed in kidneys from patients with polycystic kidney disease (Woo, 1995; Lanoix et al., 1996) and animal models of cystic disease (Ali et al., 2000). In the present study, we have used okadaic acid-induced apoptosis of RPTE cells assay to investigate the pathways involved in renal cell apoptosis. Previous studies have indicated that induction of apoptosis in renal cells with okadaic acid involves the activation of p38 MAPK pathway, however, its relationship with the caspase family has not been elucidated (Davis et al., 1996; Davis and Carbott, 1999).

The present study confirms previous reports that the p38 MAPK pathway and caspase pathway are indeed involved in apoptosis (Toyoshima et al., 1997; Juo et al., 1997; Ozaki et al., 1999; Ono and Han, 2000; Galan et al., 2000). Our data indicate that, in renal cells apoptosis, caspase-9 is activated earlier than caspases-3 and -7, consistent with the idea that caspase-9 triggers a cascade of caspase activation (Slee et al., 1999). This caspase cascade appears to be activated downstream of the p38 MAPK activation; thus, activation of p38 kinase was an earlier event and was not suppressed by several caspase inhibitors, whereas the activation of caspase-9 was completely abolished by a specific p38 kinase inhibitor SB203580 (Fig. 9). In addition, caspase activation appears to be dependent on the activation of p38 signaling pathway and other members of the MAPK, since both SB203580 and PD98059 abolished the activation of caspase-9, the first activated caspase in the cascade leading to apoptosis. A role for MAPK in apoptosis is consistent with observation that okadaic acid induces apoptosis of real epithelial cells via a mechanism that appears to involve the modulation of c-raf-1 and p38 kinase cascades. Using benzoquinone ansamycins and tyrosine kinase inhibitors geldanamycin and herbimycin A, they have examined the contribution of tyrosine phosphorylation and c-raf-1 activities to okadaic acid-induced apoptosis. Davis and Carbott (1999) showed that both geldanamycin and herbimycin A protected NRK-52E cells from okadaic acid-induced apoptosis, abrogated the overall okadaic acid-induced kinase activation, and specifically inhibited the activation of p38 kinase by okadaic acid.

The role of the MEK/ERK kinase cascade in okadaic acid-induced apoptosis is less clear. While PD98059 was able to prevent okadaic acid-induced cleavage of caspase-9, it did not prevent apoptosis (Galan et al., 2000). Thus, ERK might be involved in caspase-9 activation but okadaic acid may not require caspase-9 to cause apoptosis. This suggests that okadaic acid can induce apoptosis at several points along the caspase cascade bypassing mechanisms involving ERK and caspase-9. In contrast, p38 is downstream of all apoptotic mechanisms initiated by okadaic acid.

Our data also support the hypothesis that proteolytic cleavage of β-catenin by caspases is an important downstream molecular event associated with the morphological and cytoskeletal changes observed during apoptosis. The caspase inhibitor Z-VAD-fmk blocked the cleavage of the caspase substrate β-catenin and the apoptotic features such as DNA fragmentation, caspase-3 and -7 activity induction, and disintegration of cell membranes. Previous studies have demonstrated that aspartic residues 161/164, 144/145 located at the amino-terminal domain of β-catenin that bind to α-catenin are cleaved by caspase-3 (Brancolini et al., 1997). We have demonstrated that caspase-3 and -7 are also induced in our system, and inhibition of proteosome activity with the inhibitor lactacystin did not prevent β-catenin cleavage, indicating the cleavage was not mediated by the ubiquitin/proteosome pathway. Most importantly, cleavage of β-catenin was abolished by the caspase inhibitor Z-VAD-fmk, strongly supporting the involvement of caspases. Since β-catenin cleaved at the amino-terminal has been shown to lose its ability to bind α-catenin, disruption of binding between cadherins and the actin cytoskeleton complex is likely to contribute to the loss of cell–cell adhesive interactions (Brancolini et al., 1997; Fukuda, 1999). Our obser-

vation of the retraction and detachment of cells during apoptosis is consistent with this idea. Our data, however, do not rule out the possibility that β-catenin cleavage is associated with apoptosis independent of caspase activation.

Proteolytic cleavage of β-catenin at the NH_2-terminus by caspases can disrupt its function in Wnt signaling and interaction with other cellular proteins (Willert and Nusse, 1998). The NH_2-terminal domain of β-catenin is known to interact with adenomatous polyposis coli protein and regulates epithelial tubulogenesis (Pollack et al., 1997; Barth et al., 1997). β-Catenin also interacts with polycystin-1, the gene associated with the majority of polycystic kidney disease cases (Kim et al., 1999). Altered distribution of β-catenin has been observed in the cystic kidneys of bcl-2 knockout mouse (Sorenson, 1999).

In summary, we have demonstrated that while okadaic acid-induced caspase activation in RPTE cells is mediated by both p38 MAPK and MAPK kinase-1, okadaic acid-induced apoptosis is mediated by p38 but not ERK pathways. Additionally, cleavage of β-catenin may be an important consequence of this activation. Therefore, the caspase okadaic acid-induced apoptosis appears to function downstream of the p38 pathway.

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