Cell Death and Differentiation (2000) 7, 1119–1126
© 2000 Macmillan Publishers Ltd All rights reserved 1350-9047/00 $15.00
www.nature.com/cdd

G-protein signaling abnormalities mediated by CD95 in salivary epithelial cells

X-B Liu[4,8], R Masago[1,8], L Kong[1], B-X Zhang[6], S Masago[1], N Vela-Roch[1], MS Katz[2,5], C-K Yeh[2,3], GH Zhang[4], N Talal[7] and H Dang*,[1]

[1] Division of Clinical Immunology, Department of Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, TX 78229-3900, USA
[2] Division of Geriatrics and Gerontology, Department of Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, TX 78229-3900, USA
[3] Department of Dental Diagnostic Science, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, TX 78229-3900, USA
[4] Department of Pediatrics, The University of Texas Health Science Center at San Antonio, San Antonio, Texas, TX 78229-3900, USA
[5] Geriatric Research, Education and Clinical Center, South Texas Veterans Health Care System, Audie L Murphy Division, San Antonio, Texas, TX 78284, USA
[6] Research Services, South Texas Veterans Health Care System, Audie L Murphy Division, San Antonio, Texas, TX 78284, USA
[7] Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY 10019, USA
[8] The first two authors have made equal contributions to this work and should be considered as primary authors
* Corresponding author: The University of Texas Health Science Center at San Antonio, Department of Medicine, Division of Clinical Immunology, Mail Code 7874, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900, USA.
Tel: 210/567-5748; Fax: 210/567-4592; E-mail: dang@uthscsa.edu or hdang67521@aol.com

Received 12.8.99; revised 27.6.00; accepted 18.7.00
Edited by JC Reed

Abstract

Salivary epithelial cells from patients with primary Sjögren's syndrome (SS) undergo Fas-mediated apoptosis. Bcl-2 and Bcl-xL are apoptosis suppressing oncogenes. Very little is known about the role of these oncogene molecules in salivary epithelial cells. To investigate the possible prevention of salivary glandular destruction in SS by Bcl-2 and Bcl-xL, stable transfectants expressing these molecules were made from HSY cells, a human salivary epithelial cell line. HSY cells were transfected with an expression vector for human Bcl-2 or Bcl-xL. Stable transfectants were selected and apoptosis was induced by anti-Fas antibody. Apoptosis was quantified by propidium iodide staining followed by flow cytometry. Caspase activity was detected by immunohistochemical analysis and enzyme cleavage of DEVD-AMC, a fluorescent substrate. Response to carbachol, a muscarinic receptor agonist, and EGF was measured by Ca^{2+} mobilization and influx. Fas-mediated apoptosis was significantly inhibited in Bcl-2 and Bcl-xL transfectants compared to wild-type and control transfectants (empty vector). Surprisingly, caspase activity was not inhibited in Bcl-2 and Bcl-xL transfectants. Activation of the Fas pathway in the Bcl-2 and Bcl-xL transfectants by antibody also inhibited carbachol and EGF responsiveness (i.e., Ca^{2+} mobilization and/or influx) by 50–60%. This Fas-mediated inhibition of cell activation was partially or completely restored by specific peptide interference of caspase enzyme activity. The prevention of Fas-mediated apoptosis by the overexpression of Bcl-2 and Bcl-xL in salivary gland epithelial cells results in injured cells expressing caspase activity and unable to respond normally to receptor agonists. Such damaged cells may exist in SS patients and could explain the severe dryness out of proportion to the actual number of apoptotic cells seen on salivary gland biopsy. *Cell Death and Differentiation* (2000) **7**, 1119–1126.

Keywords: Sjögren's syndrome; signal transduction; CD95; Bcl-2

Abbreviations: SS, Sjögren's syndrome; FasL, Fas ligand; EGF, epidermal growth factor

Introduction

CD95 (i.e., Fas or APO-1) is a cell surface receptor expressed in many different types of cells and tissues.[1,2] When crosslinked by antibody or its physiological ligand (FasL) Fas sends an apoptotic signal to the cell.[3] This signal includes the activation of a cascade of cysteine proteases called caspases[4–7] and a rise in intracellular ceramide.[5,8,9] Fas-mediated apoptosis is inhibited by the protooncogene bcl-2[10] in a cell type-dependent fashion.[7] Fas-mediated apoptosis has been implicated in the pathogenesis of numerous diseases.[11–15] Several organ-specific autoimmune diseases including Sjögren's syndrome (SS), diabetes mellitus, and thyroiditis, demonstrate coexpression of Fas/FasL suggesting that target cell death is initiated by Fas-mediated apoptosis.[16–19]

The bcl-2 family of protooncogenes plays an important role in regulating apoptosis.[20] This family consists of both antagonists (e.g., bcl-2, bcl-xL) and agonists (e.g. bax) that cross-talk with one another through dimerization.[21] For example, the family member bid heterodimerizes with both bcl-2 and bax. When cleaved by caspase 8, bid counters the cytoprotective effect of bcl-2 and promote the proapoptotic effect of bax.[22] Any changes in this family of proteins, can alter the cell's sensitivity to an apoptotic signal.

The autoantigens that incite autoimmune disease are becoming better known. Recent evidence from several laboratories suggests that many of these antigens arise in cells undergoing apoptosis.[23–26] These apoptotic autoantigens are thought to induce several antinuclear and

antinucleoprotein antibodies seen in SLE and SS patients. We reported that SS salivary gland epithelial cells die apoptotically.[16] The same is true in NOD mice (a model for SS), but surprisingly also in NOD.*scid* mice which lack a mature immune system.[27] This unexpected result suggested a lymphocyte-independent early stage in SS patients in which the epithelial apoptotic lesion precedes and calls forth the lymphocytic autoimmune process.

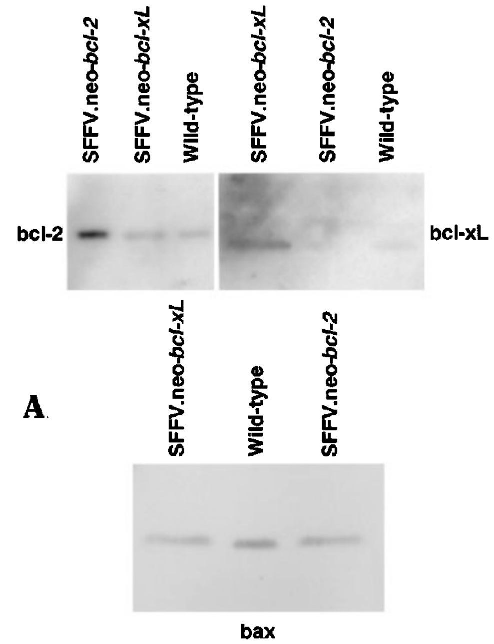
To pursue this hypothesis, we studied a human salivary gland epithelial cell line (HSY) growing in tissue culture. We induced Fas-mediated apoptosis in these HSY cells and aborted cell death by stable transfection with bcl-2 or bcl-xL. These transfected cells still manifest caspase activation and have Fas-induced defects in intracellular $[Ca^{2+}]_i$ signaling in response to carbachol and EGF. To our knowledge, this is the first report showing that the functional abnormalities associated with the Fas pathway can be segregated from apoptosis. These poorly functional cells may be a model for what occurs pathologically in SS patients where the severity of dryness is probably not solely a result of glandular destruction.

Results

Bcl-2 and Bcl-xL protect HSY cells from fas-mediated apoptosis

HSY cells were stably transfected for bcl-2 and bcl-xL expression. Transfectants only overexpressed their respective molecule as assessed by Western blot analysis (Figure 1). Furthermore, transfectants were responsive to carbachol, a muscarinic receptor agonist, and EGF (Figure 1). Sensitivity to Fas-mediated apoptosis was assessed by adding anti-Fas antibody, CH11, to cell cultures (Figure 2). After 48 h the classical DNA laddering pattern typical of apoptotic cells was seen in the empty vector transfectant but not in the bcl-2 and bcl-xL transfectant (data not shown). Further confirmation of the antiapoptotic action of bcl-2 and bcl-xL in HSY cells was obtained by quantifying apoptosis by propidium iodide staining (Figure 2A). More than 80% of the empty vector transfectants were apoptotic, whereas the bcl-2 and bcl-xL transfectants were resistant to Fas-mediated apoptosis (11 and 8% apoptotic, respectively, $P<0.05$). Furthermore, Fas-mediated apoptosis in HSY was caspase-dependent as DEVD inhibited cell death (Figure 2A). C2-ceramide, a lipid second messenger for Fas, was added to cultures. As with CH11, bcl-2 and bcl-xL transfectants were resistant to ceramide-induced apoptosis compared to the control transfectant (70 *vs* 10 and 6% apoptotic, respectively; $P<0.05$). Upregulation of bcl-2 or bcl-xL totally protected HSY cells from apoptosis and did not merely delay apoptosis. The number of apoptotic cells did not increase after 4 days in culture with CH11.

Forward light scatter (Figure 2B) showed that anti-Fas antibody did not alter cell size in the bcl-2 and bcl-xL transfectants. By contrast, cell size was reduced in the wild-type and the control transfectant after 48 h of exposure to CH-11.



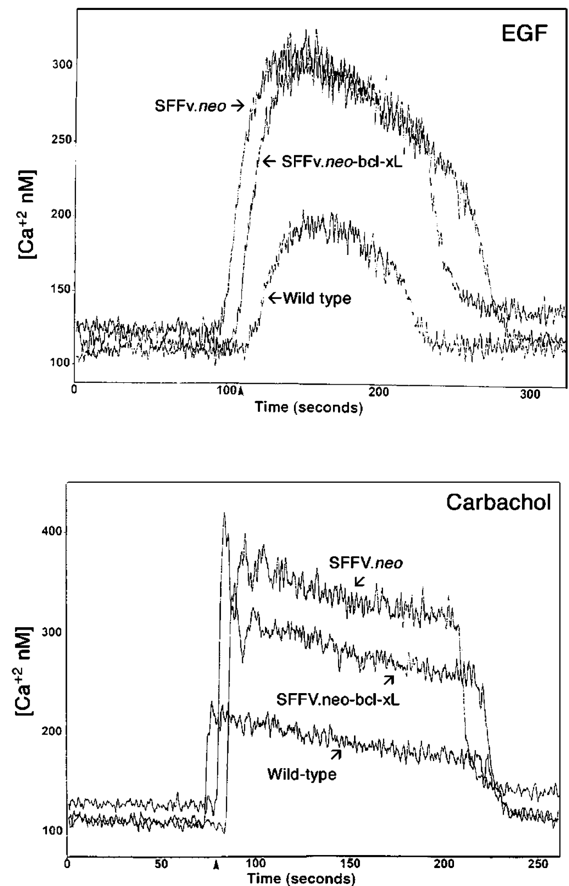
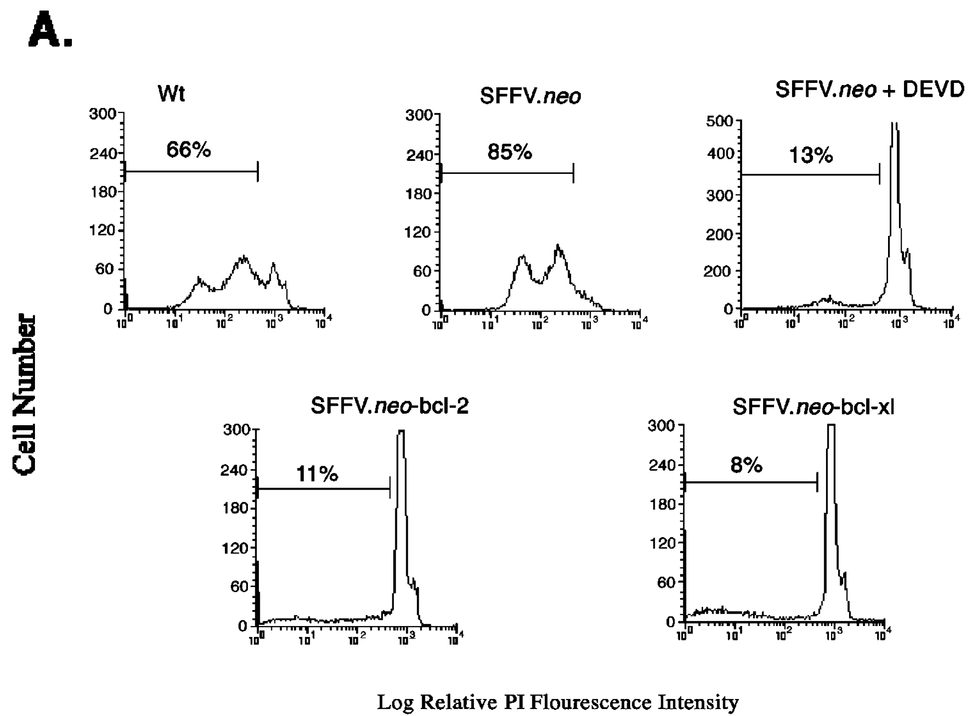


Figure 1 Bcl-2 family expression and function in HSY transfectants. Cell lysates from HSY transfectants were assessed for bcl-2, bcl-xL, and bax expression by Western blot analysis (**A**). Overexpression of bcl-2 and bcl-xL were only observed in HSY cells transfected with pSFFV-bcl-2 and pSFFV-bcl-xL, respectively. The expression of bax was not altered in the three transfectants. Fluorometric analysis was used to measure $[Ca^{2+}]_i$ in Fura-2AM-loaded HSY cells, wild-type and transfectants, stimulated with carbachol (10 μM) and EGF (50 ng/ml) (**B**). Transfectants retained their ability to respond to receptor agonists



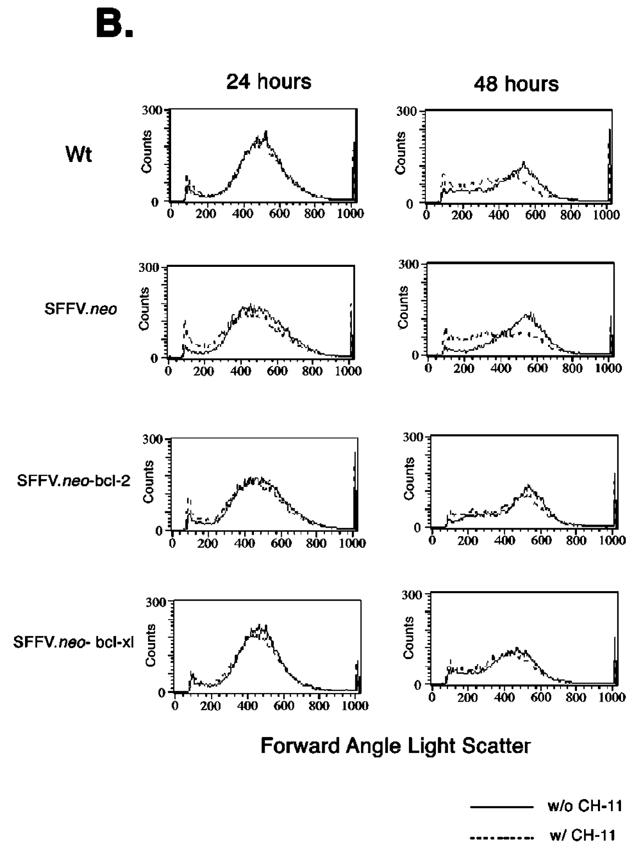


Figure 2 Resistance of transfectants to Fas-mediated apoptosis. Anti-Fas antibody, CH11 (1 μg/ml), was added to cultures for 48 h. Nuclei were stained with propidium iodide and analyzed by flow cytometry (**A**). Cells staining subdiploid were defined as apoptotic. The percentage represents [experimental – background] where the background was 3.0 – 8.7%. DEVD (100 μM) was added to cultures to inhibit caspase activation. Forward angle light scatter was performed (**B**). After 24 h with CH11, no change in cell size was observed in all of the cell lines. After 48 h of CH11 treatment, cell shrinkage was observed in the wild-type and control transfectant

Fas-mediated signaling abnormalities in Bcl-2 and Bcl-xL transfectants

Transfectants were treated with CH11 (anti-Fas antibody) and assessed for cell function by measuring intracellular free $[Ca^{2+}]_i$ in response to carbachol and EGF (Figure 3). After 24 h, a time when nuclear condensation, DNA fragmentation, and cell shrinkage was not observed, control transfectants showed decreased responses to carbachol and EGF compared to cultures with no anti-Fas antibody. Although the bcl-xL transfectant was resistant to Fas-mediated apoptosis it was nevertheless defective in response to carbachol and EGF following exposure to CH11. The abnormality in Ca^{2+} release from intracellular stores was not observed in the bcl-2 transfectants. However, C2-ceramide significantly inhibited carbachol responses in all three transfectants (Figure 4).

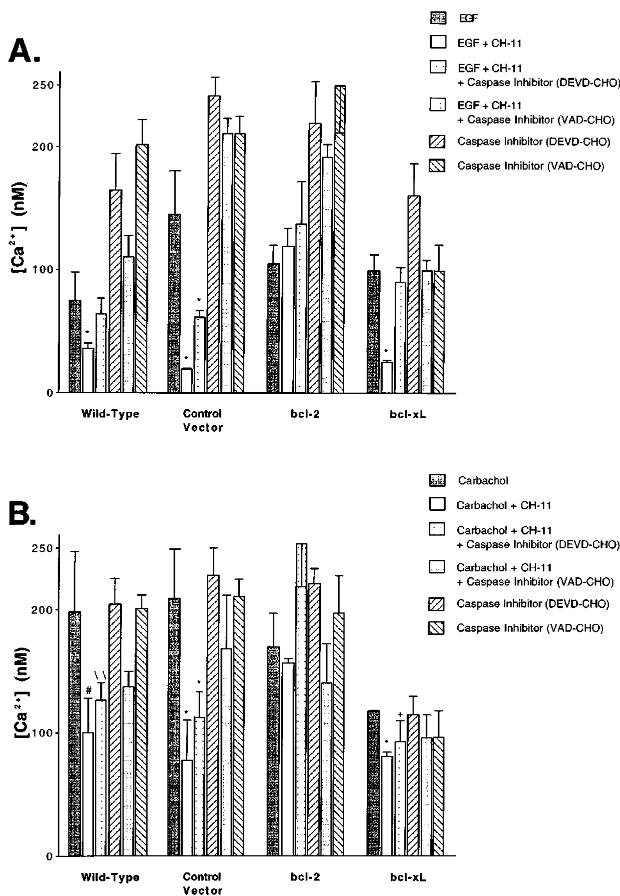


Figure 3 Fas-mediated inhibition of carbachol and EGF signal transduction in HSY transfectants. Anti-Fas antibody, CH11 (1 μg/ml), was added to cultures for 24 h. Fluorometric analysis was used to measure $[Ca^{2+}]_i$ in Fura-2AM-loaded HSY cell transfectants stimulated with carbachol (**A**) and EGF (**B**). DEVD (100 μM) or VAD (100 μM) was added to cultures to inhibit caspase activation. The presence of CH-11 inhibited the rise in $[Ca^{2+}]_i$ in all the wild-type HSY, and the control and bcl-xL transfectants. DEVD or VAD restored carbachol signal transduction to varying levels. Cells were equilibrated in Ca^{2+} containing media. Columns represent the mean $\pm$ S.D. of separate experiments (*n*=5). $^{+}P<0.05$; $^{\|}P<0.02$; $^{\#}P<0.01$; $^{*}P<0.001$ compared to cultures with either carbachol (**A**) or EGF (**B**) alone

Extracellular Ca^{2+} influx is another measurement of signal transduction. Anti-Fas antibody decreased in Ca^{2+} influx by 30–50% in all three transfectants (Figure 5). Fas-induced inhibition of Ca^{2+} influx was not affected by the overexpression of either bcl-2 and bcl-xL.

Caspase 3 is activated in the Fas-stimulated Bcl-2 and Bcl-xL transfectants

Fas stimulation leads to the activation of a cascade of cysteine proteases called caspases.[28] The caspase inhibitor DEVD or VAD was added to cultures and the transfectants

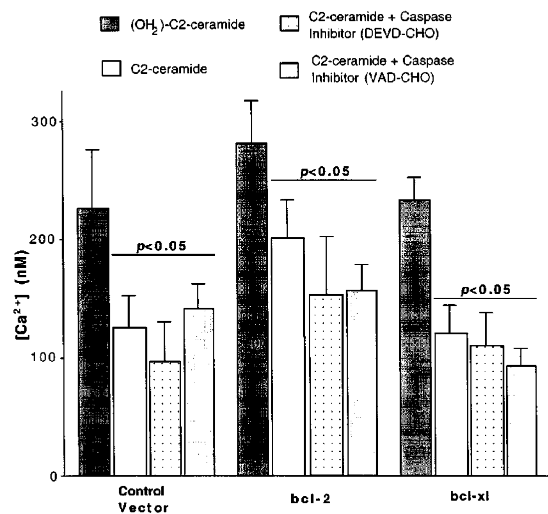


Figure 4 Ceramide-mediated inhibition of carbachol signal transduction in HSY transfectants. C2-ceramide (100 μM) was added to cultures for 6 h. Fluorometric analysis was used to measure $[Ca^{2+}]_i$ in Fura-2AM-loaded HSY cell transfectants stimulated with carbachol. DEVD (100 μM) or VAD (100 μM) was added to cultures to inhibit caspase activation. Controls are cells incubated with $(OH)_2$-C2-ceramide. The presence of C2-ceramide inhibited the rise in $[Ca^{2+}]_i$ in all three transfectants. Neither DEVD nor VAD restored carbachol signal transduction. Columns represent the mean $\pm$ S.D. of separate experiments (*n*=5). Cells were equibrated in Ca^{2+} containing media

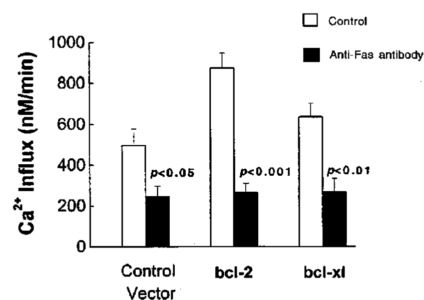


Figure 5 Fas-mediated inhibition of carbachol Ca^{2+} influx in HSY transfectants. Anti-Fas antibody, CH11 (1 μg/ml), was added to cultures for 24 h. Fluorometric analysis was used to measure Ca^{2+} influx in Fura-2AM-loaded HSY cell transfectants stimulated with carbachol. Studies were done in Ca^{2+} free media. Columns represent the mean $\pm$ S.D. of separate experiments (*n*=5)

assessed for intracellular $[Ca^{2+}]_i$ response to carbachol and EGF (Figure 3). The presence of DEVD partially restored intracellular signaling in the control transfectant and wild-type HSY. Whereas, DEVD countered the inhibitory effect of CH-11 and restored responsiveness to carbachol and EGF in the bcl-xL transfectants. The presence of VAD was much more effective in restoring carbachol and EGF responsiveness in all of the cell lines. Indeed, the presence of DEVD and VAD heightened the carbachol and EGF response.

DEVD and VAD were used to counter the inhibitory effect of ceramide (Figure 4). The presence of these two caspase inhibitors had no effect. This suggest that Fas-mediated inhibition of cell activation was not initiated by the generation of ceramide.

Immunohistochemical and enzymatic analysis was performed (Figures 6 and 7, respectively) to further demonstrate caspase activation in the absence of apoptosis. After 24 h with anti-Fas antibody, all three transfectants stained positive for activated caspase 3. Furthermore, caspase activity was quantified in cell lysates from transfectants (Figure 7). Transfectants were treated with anti-Fas antibody (CH11) or C2-ceramide, and lysates were assessed for cleavage of DEVD-AMC by fluorometry. After 24 h, CH11 and C2-ceramide induced 8–12 times as much caspase activity in the control transfectant compared to untreated cells. Similar levels of caspase activation was seen in the bcl-2 and bcl-xL transfectants.

Discussion

These experiments were undertaken to study the role of salivary gland epithelial cell apoptosis and its inhibition in the pathogenesis of SS. Based on our previous studies in SS patients we show that anti-Fas antibody induces apoptosis in a human salivary gland cell line (HSY). We found that enforced expression of the suppressor protooncogene bcl-2 and bcl-xL largely prevented HSY epithelial cells from undergoing Fas-mediated apoptosis, although caspase enzymes were activated. The rescued cells showed decreased $[Ca^{2+}]_i$ release and/or Ca^{2+} influx in response to carbachol and EGF following the activation of the Fas pathway. Signal transduction suppression by Fas has been reported in T lymphocytes prior to the onset of apoptosis (i.e. decreased nuclear content).[29] Thus bcl-2 and bcl-xL can abort Fas-mediated apoptosis but leave these damaged cells functionally abnormal. This system may provide an *in vitro* model for SS, where the severe dryness cannot be totally explained by actual number of apoptotic acinar cells found in the gland.[16,30] It is interesting to note that nonapoptotic mechanisms may also explain the salivary dysfunction in irradiated rat salivary glands.[31]

Bcl-2 and bcl-xL represent a family of homologous proteins that regulate several apoptotic pathways.[20] These proteins act at the site of the mitochondria to maintain membrane potential. The partial restoration of $[Ca^{2+}]_i$ cell signaling in the bcl-2 and bcl-xL transfectants can be attributed to the control the mitochondria exert over endoplasmic reticulum $[Ca^{2+}]_i$.[32] At the mitochondrial level bcl-2 and bcl-xL can regulate the activation of caspases.[33] The regulation of caspase activity by bcl-2 or bcl-xL is cell type-dependent.[7] In the B cell line, SKW6, overexpression of bcl-2 or bcl-xL inhibited Fas-mediated cell death but not caspase 3 activation, similar to what we find with HSY cells where caspase 3-like activation occurs in the absence of apoptosis. The lymphoblastoid T cell line JURKAT transfected with bcl-xL was resistant to Fas-mediated apoptosis.[34] Nevertheless, PARP was cleaved in these

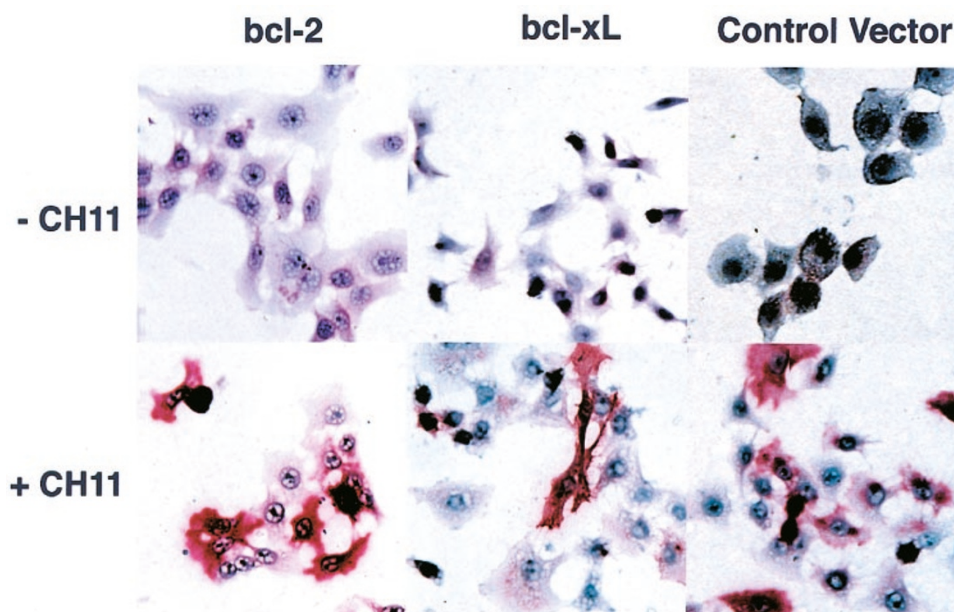


Figure 6 Fas-mediated caspase 3 cleavage in transfected HSY cells. Anti-Fas antibody, CH11 (1 μg/ml), was added to cultures for 24 h. HSY transfectants were assessed by immunohistochemistry for activated caspase 3 (magnification=250 $\times$)

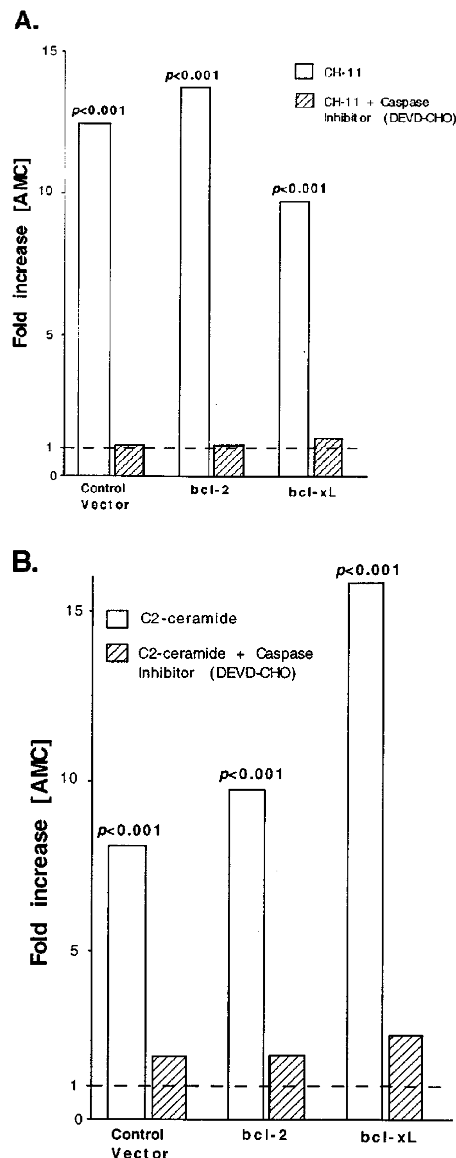


Figure 7 Fas- and ceramide-mediated DEVD-AMC cleavage in transfected HSY cells. Anti-Fas antibody, CH11 (**A**, 1 μg/ml), or C2-ceramide (**B**, 100 μM) was added to cultures for 24 h. Cell lysates were incubated with DEVD-AMC. Fluorometric analysis was used to measure free AMC. DEVD (100 μM) was added to cultures to inhibit caspase activation. Data are expressed as fold increase of AMC release compared to untreated (**A**) or $(OH)_2$-C2-ceramide treated (**B**) cells represented by (- - - -). S.D. was <3%

cells. Moreover, caspase 3 or 8 activation in the absence of apoptosis has been observed in activated cells.[35–37]

Increase in $[Ca^{2+}]_i$ is related to fluid secretion from salivary glands epithelial cells.[38] In salivary acinar cells, stimulation of the muscarinic receptor results in activation of phospholipase C and the subsequent hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-triphosphate. The former is an endogenous activator of protein kinase C which results in a rapid release of Ca^{2+} from intracellular stores.[39] EGF receptor is a transmembrane protein tyrosine kinase. Binding of EGF lead to receptor dimerization, autophosphorylation, and recruitment of kinase substrates. Subsequent events include Ras (GTP-binding protein) phosphorylation and activation of the Ras/Raf/MAP kinase pathway.[40] Like the muscarinic receptor, the EGF receptor can activate the phosphatidylinositol pathway resulting in the activation of protein kinase C and a rise in $[Ca^{2+}]_i$ and Ca^{2+} influx.[41] Fas signaling may affect both common and distinct pathways in these two receptor systems.

The Fas-mediated signaling abnormalities in HSY transfectants were partly or completely restored by caspase inhibitors, suggesting the involvement of proteolysis in this abnormality. Certain transcription factors which arise during cell activation are susceptible to proteolysis by caspases.[42,43] Apoptotic enterocytes downregulate molecules involved in Ras signaling.[44] These include the disappearance of the EGF receptor and the guanine nucleotide exchangers, Sos-1 and Sos-2. In that work the addition of a caspase inhibitor prevented the disappearance of these signal transduction molecules. Similar mechanisms may be operating in HSY cells.

There may also be caspase-independent mechanisms present in HSY cells, even in the bcl-2 and bcl-xL transfectants. For example, ceramide generation may play a role. This lipid second messager for Fas can disrupt electron transport and ATP synthesis without perturbing mitochondrial membrane potential.[45,46] Fas stimulation can also lead to c-Jun kinase activity, perhaps through the Daxx adapter molecule,[47] which may send a negative regulatory signal that interferes with carbachol and EGF signaling. Loss of glucose transport is another early event in Fas-mediated apoptosis that can disrupt normal signal transduction.[48]

The current study suggests that Fas can also disrupt normal cell signaling without inducing apoptosis. The disassociation between apoptosis and abnormal cell signaling provides a new model for disease pathogenesis. Based on our work in NOD mice and patients, Fas/FasL interaction occurs abnormally in the salivary gland.[16,27] This model in which inappropriate expression of Fas or FasL of target cells may also apply to other organ-specific autoimmune diseases such as Hashimoto's thyroiditis[13] and insulin-dependent diabetes mellitus.[49]

Materials and Methods

Cell preparation

HSY is an adenocarcinoma cell line derived from an acinar-intercalated duct region of a human parotid gland.[50] The HSY cell line was developed by Dr. Patton (NIH) and kindly provided by Dr. JT Turner (NIDCR, NIH). Cells were cultured in Dulbecco's Modified Eagle's medium (Gibco BRL, Gaithersburg, MD, USA), 10% FCS (Gibco BRL), 10% L-glutamine (Gibco BRL) and penicillin-streptomycin (Gibco BRL), 6% CO_2. Transfected cells were cultured in medium as described but containing 500 μg/ml Geneticin (Gibco BRL).

Antibodies and reagents

Mouse monoclonal anti-Fas antibody, CH11, was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). Mouse

monoclonal antibodies to bax, bcl-2 and bcl-xL were purchased from Trevigen Inc (Gaithersburg, MD, USA). Alkaline phosphatase-conjugated streptavidin and 7-amino-4-methylcoumarin (AMC) were from Sigma Chemical Co. (St Louis, MO, USA). Ac-DEVD-AMC, a caspase-3 (cpp 32) fluorogenic substrate, rabbit antibody to activated caspase 3, and APO-BRDU™ kit were purchased from PharMingen (San Diego, CA, USA). LipofecTAMINE™ reagent was purchased from Gibco BRL Life Technologies, Inc (Gaithersburg, MD, USA). Z-Asp-Glu-Val-Asp-CH2F (DEVD) and Z-Val-Ala-Asp-CH2F (VAD) was from Enzyme System Products Inc (Dublin, CA, USA). C2-ceramide and $(OH)_2$-C2-ceramide, a biologically inert form of C2-ceramide, was purchased from CalBiochem (La Jolla, CA, USA).

Stable cell transfection

Plasmids pSFFV-neo (the empty vector), pSFFV.neo-Bcl-2 and pSFFV.neo-Bcl-xL were kind gifts from Dr. C Thompson[10] (University of Chicago Medical Center). HSY cells were grown to 80% confluency and rinsed with serum-free medium followed by the addition of a mixture of plasmid (1 μg) and lipofecTAMINE™ (10 μl) in serum-free medium. After 18 h at 37°C, FCS was added to a final concentration of 10% for 6 h. Transfectants were selected for drug resistance with Geneticin (500 μg/ml). Resistant colonies were transferred to tissue culture plates. Western blot analysis was used to assess over-expression of bcl-2 or bcl-xL of isolated clones.

Propidium iodide staining

Cells (1×10^6) were resuspended in hypotonic buffer containing propidium iodide (50 μg/ml, 0.1% sodium citrate, 0.1% Triton X-100) overnight at 4°C in the dark as described by Nicoletti *et al*.[51] DNA content was quantified by flow cytometry. Cells in the hypodiploid region were defined as apoptotic.

DNA fragmentation

DNA was extracted from ten million cells using the Trevigen DNA laddering kit (Gaithersburg, MD, USA). DNA was separated by gel electrophoresis in 3% agarose and visualized by ethidium bromide staining.

Immunohistochemical assessment

Transfectants were fixed with 4% paraformaldehyde and washed in Tris buffered saline (pH 7.6). Rabbit antibody specific for the activated form of caspase 3 (1 : 40 v/v) as added to the fixed cells for 30 min at room temperature. A peroxidase immunoconjugate was subsequently added (1 : 100 v/v) followed by biotinyl tyramide (1 : 50 v/v) and alkaline phosphatase-conjugated streptavidin. Color development was achieved with Vector Red (Vector Laboratories, Burlingame, CA, USA) and counterstained with hematoxylin.

Detection of caspase activation

Cells were incubated with anti-Fas antibody or C2-ceramide for 24 h and resuspended in lysis buffer (10 mM Tris, pH 7.5, 130 mM NaCl, 1% Triton X-100) on ice for 5 min. One hundred μg of cell lysates was incubated with 20 μM of Ac-DEVD-AMC in 1 ml of 20 mM HEPES (pH 7.5, 10% glycerol, 2 mM DTT) for 2 h at 37°C. A spectro-fluorometer was used to measure liberated AMC from Ac-DEVD-AMC using an excitation wavelength of 380 nm and an emission wavelength of 445 nm. A standard curve using AMC was used to quantify caspase activity.

Intracellular $[Ca^{2+}]_i$ and Ca^{2+} influx

$[Ca^{2+}]_i$ and Ca^{2+} influx was quantified by spectrofluorometry using Fura-2AM (Molecular Probes, Eugene, OR, USA) as the fluorescent dye. Cells were loaded with 2 μM of Fura-2Am for 45 min at 30°C.[52] Fluorescent wavelength measurements were set at 340 and 380 nm excitation, and 510 nm emission. Readings were done on a PTI Systems Delta Scan spectrofluorometer (Photo Technology International Inc., South Brunswick, NJ, USA). Cytosolic free $[Ca^{2+}]_i$ was calculated according to Vandenberghe *et al*.[53] After a basal level of free $[Ca^{2+}]_i$ was established, cells were stimulated by the addition of carbachol (10 μM final concentration) or EGF (50 ng/ml final concentration). Readings were done in either media containing Ca^{2+} (100 mM) or were Ca^{2+}-free. The latter was required to measure Ca^{2+} influx.

Statistical analysis

Nonparametric Student's *t*-test was used. $P<0.05$ was considered statistically significant.

Acknowledgments

The authors are grateful to Drs. Rudin and Lee for their advice and help in establishing stable transfectants; and Charles Thomas for flow cytometric analysis. Support for this work was provided by grants from the RGK Foundation (Austin, TX) and National Institute of Dental and Craniofacial Research (DE10863 and DE12203 to H Dang; DE90270 to GH Zhang; DE12188 to C-K Yeh).

References

1. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima SI, Sameshima M, Hase A, Seto Y and Nagata S (1991) The polypeptide encoded by the cDNA for human cell surface antigen fas can mediate apoptosis. Cell 66: 233–243
2. Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, Li-Weber M, Richards S, Dheim J, Trauth BC, Ponsting H and Krammer PH (1992) Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. J. Biol. Chem. 267: 10709–10715
3. Nagata S and Golstein P (1995) The Fas death factor. Science 267: 1449–1456
4. Enari M, Hug H and Nagata S (1995) Involvement of an ICE-like protease in Fas-mediated apoptosis. Nature 375: 78–81
5. Cifone MG, Roncaioli P, De Maria R, Camarda G, Santoni A, Ruberti G and Testi R (1995) Multiple pathways originate at the Fas/APO-1 (CD95) receptor: sequential involvement of phosphatidylcholine-specific phospholipase C and acidic sphingomyelinase in the propagation of the apoptotic signal. EMBO J. 14: 5859–5868
6. Los M, Van de Craen M, Penning LC, Schenk H, Westendorp M, Baeuerle PA, Dröge W, Krammer PH, Fiers W and Schulze-Osthoff K (1995) Requirement of an ICE/CED-3 protease for Fas/APO-1-mediated apoptosis. Nature 375: 81–83
7. Scaffidi CS, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin K-M, Krammer PH and Peter ME (1998) Two CD995 (APO-1/Fas) signaling pathways. EMBO J. 17: 1675–1687
8. Gill BM, Nishikata H, Chan G, Delovitch TL and Ochi A (1994) Fas antigen and sphingomyelin-ceramide turnover-mediated signaling: role in life and death of T lymphocytes. Immunol. Rev. 142: 113–145
9. Cifone MG, De Maria R, Roncaioli P, Rippo MR, Azuma M, Lanier LL, Santoni A and Testi R (1994) Apoptotic signaling through CD95 (Fas/Apo-1) activates an acidic sphingomyelinase. J. Exp. Med. 180: 1547–1552

10. Boise LH, Gonzalez-Gracia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nuñez G and Thompson CB (1993) *bcl-x*, a *bcl-2*-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74: 597–608
11. Wilson SE, Li Q, Weng J, Barry-Lane PA, Jester JV, Liang Q and Wordinger RJ (1996) The Fas-Fas ligand system and other modulators of apoptosis in the cornea. Invest. Ophthalmol. Vis. Sci. 37: 1582–1592
12. Galle PR, Hofmann WJ, Walczak H, Schaller H, Otto G, Stremmel W, Krammer PH and Runkel L (1995) Involvement of the CD95 (APO-1/Fas) receptor and ligand in liver damage. J. Exp. Med. 182: 1223–1230
13. Giordano C, Stassi G, D Maria R, Todaro M, Richiusa P, Papoff G, Ruberti G, Bagnasco M, Testi R and Galluzzo A (1997) Potential involvement of Fas and its ligand in the pathogenesis of Hashimoto's thyroiditis. Science 275: 960–963
14. Kondo T, Suda T, Fukuyama H, Adachi M and Nagata S (1997) Essential roles of the Fas ligand in the development of hepatitis. Nat. Med. 4: 409–413
15. Schelling JR, Nkemere N, Kopp J and Cleveland RP (1998) Fas-dependent fratricidal apoptosis is a mechanism of tubular epithelial cell deletion in chronic renal failure. Lab. Invest. 78: 813–824
16. Kong L, Ogawa N, Nakabayashi T, Liu GT, D'Souza E, McGuff HS, Guerrero D, Talal N and Dang H (1997) *Fas* and *Fas* ligand expression in salivary glands of patients with primary Sjögren's syndrome. Arthritis Rheum. 39: 87–97
17. Nakajima T, Aono H, Hasunuma T, Yamamoto K, Shirai T, Hirohata K and Nishioka K (1995) Apoptosis and functional Fas antigen in rheumatoid arthritis synoviocytes. Arthritis Rheum. 38: 485–491
18. Dowling P, Shang G, Raval S, Menonna J, Cook S and Husar W (1996) Involvement of the CD95 (APO-1/Fas) receptor/ligand system in multiple sclerosis brain. J. Exp. Med. 184: 1513–1518
19. De Maria R and Testi R (1998) Fas-FasL interactions: a common pathogenetic mechanism in organ-specific autoimmunity. Immunol. Today 19: 121–125
20. Green DR and Reed JC (1998) Mitochondria and apoptosis. Science 281: 1309–1312
21. Oltvai ZN and Korsmeyer SJ (1994) Checkpoints of dueling dimers foil death wishes. Cell 79: 189–192
22. Wang K, Yin XM, Chao DT, Milliman CL and Korsmeyer SJ (1996) BID: a novel BH3 domain-only death agonist. Genes Dev. 10: 2859–2869
23. Casciola-Rosen LA, Anhalt G and Rosen A (1994) Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. J. Exp. Med. 179: 1317–1330
24. Tan EM (1994) Autoimmunity and apoptosis. J. Exp. Med. 179: 1083–1086
25. Casciola-Rosen L, Rosen A, Petri M and Schlissel M (1996) Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythemeatosus. Proc. Natl. Acad. Sci. USA 93: 1624–1629
26. Pittoni V and Isenberg D (1998) Apoptosis and antiphospholipid antibodies. Sem. Arthritis Rheum. 28: 163–178
27. Kong L, Robinson CP, Peck AB, Vela-Roch N, Sakata KM, Dang H, Talal N and Humphreys-Beher MG (1998) Inappropriate apoptosis of salivary and lacrimal gland epithelium of immunodeficient NOD-scid mice. Clin. Exp. Rheumatol. 16: 675–681
28. Ji L, Ito M, Zhang G, Hirabayashhi Y, Inokuchi J and Yamagata T (1998) Effects of endoglycoceramidase of D-threo-1-phenyl-2-decanoylamino-3 morpholino-1-propanol on glucose uptake, glycolysis, and mitochondrial respiration in HL60 cells. Arch. Biochem. Biophys. 359: 107–114
29. Yang X, Khosravi-Far R, Chang HY and Baltimore D (1997) Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell 89: 1067–1076
30. Berridge MV, Tan AS, McCoy KD, Kansara M and Rudert F (1996) CD95 (Fas/Apo-1)-induced apoptosis results in loss of glucose transporter function. J. Immunol. 156: 4092–4099
31. Sainio-Pollanen S, Erkkila S, Alanko S, Hanninen A, Pollanen P and Simell O (1998) The role of Fas ligand in the development of insulitis in nonobese diabetic mice. Pancreas 16: 154–159
32. Martin SJ and Green DR (1995) Protease activation during apoptosis: death by a thousand cuts. Cell 82: 349–352
33. Kovacs B and Tsokos GC (1995) Cross-linking of the Fas/Apo-1 antigen suppresses the CD3-mediated signal transduction events in human T lymphocytes. J. Immunol. 155: 5543–5549
34. Kong L, Ogawa N, McGuff HS, Nakabayashi T, Sakata K-M, Masago R, Vela-Roch N, Talal N and Dang H (1998) Bcl-2 family expression in salivary glands from patients with primary Sjögren's syndrome: involvement of bax in salivary gland destruction. Clin. Immunol. Immunopathol. 88: 133–141
35. Paardekooper GM, Cammelli S, Zeilstra LJ, Coppes RP and Konings AW (1998) Radiation-induced apoptosis in relation to acute impairment of rat salivary gland function. Int. J. Radiat. Biol. 73: 641–648
36. Hoth M, Fanger CM and Lewis RS (1997) Mitochondrial regulation of store operated calcium signaling in T lymphocytes. J. Cell. Biol. 137: 633–648
37. Susin SA, Zamzami N, Castedo M, Hirsch T, Marchetti P, Macho A, Daugas E, Geusken M and Kroemer G (1996) Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. J. Exp. Med. 184: 1331–1341
38. Boise LH and Thompson CB (1997) Bcl-x(L) can inhibit apoptosis in cells that have undergone Fas-induced protease activation. Proc. Natl. Acad. Sci. USA 94: 3759–3764
39. Miossec C, Dutilleul V, Fassey F and Diu-Hercend A (1997) Evidence for CPP32 activation in the absence of apoptosis during T lymphocyte stimulation. J. Biol. Chem. 272: 13459–13462
40. Wilhelm S, Wagner H and Häcker G (1998) Activation of caspase-3-like enzymes in non-apoptotic T cells. Eur. J. Immunol. 28: 891–900
41. Kennedy NJ, Kataoka T, Tschopp J and Budd RC (1999) Caspase activation is required for T cell proliferation. J. Exp. Med. 190: 1891–1896
42. Puney Jr JW (1986) Identification of cellular activation mechanism associated with salivary secretion. Annu. Rev. Physiol. 50: 75–88
43. Ambudkar IS, Hiramatsu Y, Lockwich T and Baum BJ (1993) Activation and regulation of calcium entry in rat parotid gland acinar cells. Crit. Rev. Oral Biol. Med. 4: 421–425
44. Teitebaum I (1990) The epidermal growth factor receptor is coupled to a phospholipase A2-specific pertussis toxin-inhibitable guanine nucleotide-binding regulatory protein in cultured rat inner medillary collecting tubule cells. J. Biol. Chem. 265: 4218–4222
45. Pai R and Tarnawski A (1998) Signal transduction cascades triggered by EGF receptor activation: relevance to gastric injury repair and ulcer healing. Dig. Dis. Sci. 43: 14S–22S
46. King P and Goodburn S (1998) STAT1 is inactivated by a caspase. J. Biol. Chem. 273: 8699–8794
47. Ravi R, Bedi A, Fuchs EJ and Bedi A (1998) CD95 (Fas)-induced caspase-mediated proteolysis of NF-kappaB. Cancer Res. 58: 882–886
48. Scheving LA, Jin W-H, Chong K-M, Gardner W and Cope FO (1998) Dying enterocytes downregulate signaling pathways converging on Ras: rescue by protease inhibition. Am. J. Physiol. 274: C1363–C1372
49. France-Lanord V, Brugg B, Michel PP, Agid Y and Ruberg M (1997) Mitochondrial free radical signal in ceramide-dependent apoptosis: a putative mechanism for neuronal death in Parkinson's disease. J. Neurochem. 69: 1612–1621
50. Patton L, Pollack S and Wellner R (1991) Responsiveness of a human parotid epithelial cell line (HSY) to autonomic stimulation: muscarinic control of K^+ transport. *In vitro* Cell. Dev. Biol. 27A: 779–785
51. Nicoletti I, Migliorati G, Pagliacci MC, Grigani F and Riccardi C (1991) A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Meth. 139: 271–279
52. Olsen JG, Salih MA, Harrison JL, Herrera I, Luther MF, Kalu DN, Lifschitz MD, Katz MS and Yeh C-K (1997) Modulation by food restriction of intracellular calcium signaling in parotid acinar cells of aging Fischer 344 rats. J. Gerontol. 52A: B152–B158
53. Vandenberghe PA and Ceuppens JL (1990) Flow cytometric measurement of cytoplasmic free calcium in human peripheral blood T lymphocytes with fluo-3, a new fluorescent calcium indicator. J. Immunol. Meth. 127: 197