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The inhibition of human T cell proliferation by the caspase inhibitor z-VAD-FMK is mediated through oxidative stress

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ABSTRACT

The caspase inhibitor benzyloxycarbony (Cbz)-L-Val-Ala-Asp (OMe)-fluoromethylketone (z-VAD-FMK) has recently been shown to inhibit T cell proliferation without blocking caspase-8 and caspase-3 activation in primary T cells. We showed in this study that z-VAD-FMK treatment leads to a decrease in intracellular glutathione (GSH) with a concomitant increase in reactive oxygen species (ROS) levels in activated T cells. The inhibition of anti-CD3-mediated T cell proliferation induced by z-VAD-FMK was abolished by the presence of low molecular weight thiols such as GSH, N-acetylcysteine (NAC) and L-cysteine, whereas D-cysteine which cannot be metabolised to GSH has no effect. These results suggest that the depletion of intracellular GSH is the underlying cause of z-VAD-FMK-mediated inhibition of T cell activation and proliferation. The presence of exogenous GSH also attenuated the inhibition of anti-CD3-induced CD25 and CD69 expression mediated by z-VAD-FMK. However, none of the low molecular weight thiols were able to restore the caspase-inhibitory properties of z-VAD-FMK in activated T cells where caspase-8 and caspase-3 remain activated and processed into their respective subunits in the presence of the caspase inhibitor. This suggests that the inhibition of T cell proliferation can be uncoupled from the caspase-inhibitory properties of z-VAD-FMK. Taken together, the immunosuppressive effects in primary T cells mediated by z-VAD-FMK are due to oxidative stress via the depletion of GSH.

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Introduction

Peptidyl fluoromethylketone (FMK) caspase inhibitors are very useful tools and have been used extensively in cell death research to elucidate the role of caspases during apoptotic cell death (Caserta et al., 2003; Ekert et al., 1999; Garcia-Calvo et al., 1998; Yagi et al., 2001). All peptidyl-FMK caspase inhibitors contain a peptide sequence based on the target cleavage sequence of the substrate. The family of caspases recognises a sequence of four amino acids in the substrates, designated P4-P3-P2-P1 and cleave substrates after an Asp residue at P1 (Shi, 2002; Yuan et al., 1993). All the peptide based caspase inhibitors used to date consist of a peptide sequence culminating in an Asp residue, whereas the requirements for amino acid residues at the other positions vary with members of the caspase family (Garcia-Calvo et al., 1998). By exploiting the differences in their substrate specificities, more specific inhibitors can be developed for the caspases (Garcia-Calvo et al., 1998; Thornberry et al., 1997). Conjugated to the C-terminal of the peptide sequence in the caspase inhibitor is a halomethylketone, such as FMK or chloromethylketone (CMK), which forms an irreversible covalent bond with the S-H group of the cysteine residue in the caspase active site (Caserta et al., 2003; Garcia-Calvo et al., 1998). Finally, to enhance the permeability of the inhibitor, a benzyloxycarbonyl (z) or an acetyl (Ac) group attached to the amino-terminal of the peptide sequence will increase the hydrophobicity of the compound (Van Noorden, 2001). Together, these caspase inhibitors act as competitive inhibitors by mimicking the substrates and irreversibly blocking the caspase activities.

However, accumulating evidence from a number of studies have suggested that these caspase inhibitors may not be as specific as originally envisaged. For instance the most widely used broad spectrum caspase inhibitor, z-VAD-FMK, has been shown to inhibit other enzymes besides the caspases. These include the lysosomal cysteine protease, cathepsin B (Schotte et al., 1999), peptide:N-glycanase (Misaghi et al., 2006) and picornaviral 2A proteinases (Deszcz et al., 2004). Similarly, the caspase-8 inhibitor, z-IETD-FMK, also inhibited picornaviral 2A proteinases (Deszcz et al., 2004). Some of the non-specific effects of these caspase inhibitors may account for some of the inconsistencies observed in the blocking of T cell activation and proliferation reported in several early studies (Boissonnas et al., 2002; Kennedy et al., 1999; Mack and Hacker, 2002; Zapata et al., 1998). More recently, we showed that z-VAD-FMK readily inhibits primary T cell proliferation without blocking the activation and processing of caspase-8 and caspase-3 (Lawrence and Chow, 2012). However, z-VAD-FMK is extremely effective in blocking the activation of caspases in these cells during apoptosis. Because caspase-8 plays a pivotal role in T cell activation and

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proliferation (Chun et al., 2002; Falk et al., 2004; Salmena et al., 2003), the suppression of T cell proliferation mediated by z-VAD-FMK is independent of its caspase-inhibitory properties.

In the present study we examined the molecular mechanism that underlies the immunosuppressive properties of z-VAD-FMK. Our results showed that z-VAD-FMK treatment induced depletion of intracellular GSH level in cells in a time-dependent manner with a concomitant increase in ROS level. The inhibition of mitogen-induced T cell proliferation mediated by z-VAD-FMK was readily abolished by low molecular weight thiols such as NAC, L-cysteine and GSH but not with D-cysteine. Taken together, these results suggest that the inhibition of T cell proliferation mediated by z-VAD-FMK is due to oxidative stress via the depletion of GSH.

Materials and methods

Reagents. The following chemicals were obtained from Sigma Aldrich (USA): Glutathione (GSH), L-cysteine, D-cysteine, N-acetylcysteine (NAC), L-Buthionine-S, R-sulfoximine (BSO), monochlorobimane (MCB) and dihydroethidium (DHE). Benzyloxycarbonyl-Val-Ala-Asp-(O-methyl)-fluoromethylketone (z-VAD-FMK) was purchased from ICN (USA). Monoclonal antibody (mAb) against CD3 (clone OKT3) was purified from hybridoma (ATCC) culture supernatants as previously described (Lawrence et al., 2006). Lymphoprep was from Axis-Shield PoCAS (Norway) while RPMI 1640 and FCS were from Gibco (UK). FITC-conjugated anti-CD25 and PE-conjugated anti-CD69 were acquired from BD Pharmingen (UK). The 5-bromo-2′-deoxyuridine (BrdU) labelling kit was obtained from Roche (Switzerland). Goat-anti caspase-8 and rabbit anti-caspase-3 was from Santa Cruz Biotechnology (USA) while secondary HRP-conjugated antibodies were from Dako (UK).

Peripheral mononuclear blood cell isolation. Peripheral venous blood was obtained from normal healthy volunteers, with appropriate ethical clearance, and collected into heparinised Vacutainer tubes (Becton Dickinson). Human peripheral blood mononuclear cells (PBMCs) were isolated from the red blood cells using density gradient centrifugation. In brief, the peripheral blood was diluted with RPMI and layered onto lymphoprep (density gradient of 1.077), and centrifuged at 800 ×*g* for 30 min. The PBMCs at the interface between lymphoprep and plasma were collected, washed and re-suspended in RPMI containing 10% (v/v) foetal calf serum (FCS), 10 mM L-glutamine (Invitrogen, UK), penicillin (100 U/ml) and streptomycin (100 μg/ml) until used. The viability of PBMCs was assessed using trypan blue exclusion assay and routinely determined to be >95%.

Cell proliferation assays. The proliferation of T cells following stimulation with anti-CD3 was determined using a colorimetric immunoassay based on the measurement of incorporated BrdU in the DNA during synthesis (Roche, Switzerland). The BrdU assay was performed according to the manufacturer's instructions. In brief, PBMCs (1×10^6 cells/ml) in RPMI 1640 supplemented with 10% FCS were stimulated with plate bound 5 μg/ml anti-CD3 in the absence or presence of z-VAD-FMK for various time periods in an atmosphere of 5% CO_2 in air at 37 °C. The cells were cultured for 72 h with the last 3 h pulsed with 10 μM BrdU per well. At the end of the culture period, the plates were centrifuged and cells fixed with 200 μl ethanol (70%) in HCl (final concentration 0.5 M) for 30 min at −20 °C. Following fixation, the DNA in the cells was partially digested by nuclease treatment for 30 min at 37 °C before incubating with a horse radish peroxidase-conjugated BrdU antibody. After three rounds of washing, a substrate was added and the coloured product cleaved was measured after 20 min incubation (room temperature) at 405 nm with a reference wavelength of 495 nm using a microplate reader (Tecan 200).

Determination of intracellular GSH in activated T cells. The intracellular GSH level in activated T cells was determined as described previously (Aoshiba et al., 2001; Apostolova et al., 2010; Osseni et al., 1999; Sun et al., 2009). Following treatments, the cells (1×10^4 cells) were centrifuged down at 3500 rpm for 10 min and washed with 100 μl PBS. The supernatants were carefully removed before adding 100 μl of MCB (100 μM) in PBS for 30 min at 37 °C in the dark. Unbound MCB is almost nonfluorescent, whereas the dye fluoresces blue when bound to GSH. The fluorescence in the samples was determined using a Tecan Infinite M200 fluoro-plate reader with excitation and emission wavelengths of 390 and 460 nm, respectively. A control containing media alone plus MCB was used as a blank and subtracted from the sample absorbance.

Detection of ROS in activated T cells. Intracellular ROS in activated T cells was detected by using the redox sensitive fluorescent dye, DHE. Once inside the cells DHE will be oxidised by the ROS to form ethidium, which binds the nuclear DNA and emits a red fluorescence that can be detected with a flow cytometer. Following treatments, cells (1×10^6) were washed and the cell pellet re-suspended in 1 ml of pre-warmed serum-free RPMI. DHE was added to a final concentration of 5 mM and the cells were incubated in the dark for 30 min at 37 °C, washed with ice-cold PBS before re-suspending in 1 ml of PBS prior to analysis by flow cytometry. The samples were gated to include 1×10^4 small resting T cells and large activated T cells, excluding cell debris, based on the forward- and side-scatter profiles. For the fluorescent 2-hydroxyethidium, an excitation wavelength of 532 nm (FL-2 channel) was used and the machine was calibrated using unstained cells prior to each experiment.

Western blotting. Following treatments, the PBMCs were layered over lymphoprep and centrifuged to remove the dead cells. The viable cells were washed in PBS and cell lysates prepared by three consecutive freeze–thaw cycles in an appropriate volume (10 μl per 1×10^6 cells) of lysis buffer (0.1 M NaCl, 1 mM Tris HCl at pH7.6, 1 mM EDTA, 1% Triton-X, 1 mM PMSF). The protein concentration was determined using the Bradford assay (Biorad, Germany). Protein equivalent to 20 μg whole-cell lysates was diluted in loading buffer (2% SDS, 10% Glycerol, 50 mM Tris-HCl pH 6.8, 0.2% Bromophenol Blue and 100 mM DTT) and resolved using 13% SDS-polyacrylamide gel electrophoresis. The separated proteins were transfered onto Hybond C membrane (Amersham, UK) and probed with antibodies to caspase-8 and caspase-3. Detection was carried out using chemiluminescence (Amersham). Following caspase detection, the membrane was incubated with stripping buffer before reprobing with antibodies to β-actin.

Statistical analysis of the data. The experimental data were analysed using Student's *t* test or one-way analysis of variance followed by Dunnet's test.

Results

z-VAD-FMK inhibits anti-CD3-induced T cell proliferation in PBMCs

In order to characterise the underlying mechanism of z-VAD-FMK-induced immunosuppression, the effect of z-VAD-FMK on T cell proliferation induced by anti-CD3 in PBMCs was determined. As illustrated in Fig. 1, z-VAD-FMK induced suppression of anti-CD3-mediated T cell proliferation in a concentration-dependent manner as determined by the incorporation of BrdU. Although lower concentration of z-VAD-FMK (25 μM) was less effective in blocking T cell proliferation induced by anti-CD3, significant inhibition was observed with 50 and 100 μM, which is very much in line with our previous report (Lawrence and Chow, 2012). From the results in Fig. 1, an IC_{50} of 70 μM was determined and this concentration of z-VAD-FMK was used in all subsequent experiments.

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