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Western and Chinese Antirheumatic Drug-Induced T Cell Apoptotic DNA Damage Uses Different Caspase Cascades and Is Independent of Fas/Fas Ligand Interaction

Jenn-Haung Lai, Ling-Jun Ho, Kuo-Cheng Lu, Deh-Ming Chang, Men-Fang Shaio, and Shou-Hwa Han

Spontaneous or therapeutic induction of T cell apoptosis plays a critical role in establishing transplantation tolerance and maintaining remission of autoimmune diseases. We investigated the mechanisms of apoptosis induced by Chinese and Western antirheumatic drugs (ARDs) in human T cells. We found that hydroxychloroquine, Tripterygium wilfordii hook F, and tetrandrine (Tet), but not methotrexate, at therapeutic concentrations can cause T cell death. In addition, Tet selectively killed T cells, especially activated T cells. Although ARD-induced cytotoxicity was mediated through apoptotic mechanisms, Fas/Fas ligand interaction was not required. We further demonstrated that the processes of phosphatidylserine externalization and DNA damage along the ARD-induced T cell apoptotic pathway could operate independently, and that selective inhibition of DNA damage by caspase inhibitors did not prevent T cells from undergoing cell death. Moreover, we found that Tet- and Tripterygium wilfordii hook F-induced T cell DNA damage required caspase-3 activity, and hydroxychloroquine-induced T cell DNA damage was mediated through a caspase-3- and caspase-8-independent, but Z-Asp-Glu-Val-Asp-fluomethyl ketone-sensitive, signaling pathway. Finally, the observation that ARD-induced activation of caspase-3 in both Fas-sensitive and Fas-resistant Jurkat T cells indicates that Fas/Fas ligand interaction plays no role in ARD-induced T cell apoptosis. Our observations provide new information about the complex apoptotic mechanisms of ARDs, and have implications for combining Western and Chinese ARDs that have different immunomodulatory mechanisms in the therapy of autoimmune diseases and transplantation rejection.


The etiology of autoimmune diseases, including rheumatoid arthritis, remains largely unknown. Current therapy for rheumatoid arthritis is aimed at attenuating disease activity with a combination of disease-modifying antirheumatic drugs (DMARDs), including methotrexate (MTX), sulfasalazine, and hydroxychloroquine (HCQ) (1, 2). Despite aggressive medical treatment, the morbidity and mortality of rheumatoid arthritis are still high. Therapy is limited by the limited understanding of pathophysiologic processes underlying the illness. In this context, although the activation of other immune effector cells such as macrophages, B cells, and synovial cells may participate in the process of joint destruction in rheumatoid arthritis, the process of disease initiation and progression is triggered by T cell activation (3, 4). Therefore, most therapies target the inhibition of activated T cells (5, 6).

In addition to the activation of T cells, defective regulation of T cell apoptosis (programmed cell death) also plays a crucial role in disease progression (7–9). Recent work demonstrating the requirement for T cell apoptosis to establish transplantation tolerance further highlights the importance of this process (10). Apoptosis-based therapy has been suggested as one of the approaches to controlling the progression of autoimmune diseases (11). We and other investigators showed that the immunosuppressive effects of the Western antirheumatic drug (ARD), HCQ, and of Chinese ARDs, tetrandrine (Tet) and Tripterygium wilfordii hook F (TWHf), were mediated through both the inhibition of T cell activation and the induction of T cell apoptosis (12–15). In addition, high concentrations (0.1–10 μM) of MTX, another important DMARD, have been shown to induce apoptosis of activated PBLs (16).

HCQ is considered to be an agent whose immunomodulatory effects are comparable to those of other DMARDs, but it is less toxic, and it is useful in combination therapy (17). TWHf (or Thunder God Vine, a complex herbal remedy) has been widely used, with great success clinically, to treat autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis in mainland China for decades (reviewed in Refs. 18 and 19). Purified products from TWHf have been shown to down-regulate the expression of cyclooxygenase-2, a critical molecule that is induced during inflammation (20). Aside from TWHf, the plant alkaloid Tet is another major ARD used to treat silicosis and rheumatic diseases in mainland China (21). The immunomodulatory effects of Tet and its analogs have been demonstrated both in vitro and in vivo (22–25). Recent work from us and other investigators on

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these two Chinese ARDs provide additional support for their great potential not only in the therapy of autoimmune diseases, but also in the prevention of transplantation rejection (14, 15, 26, 27).

In the present study the molecular events that mediate ARD-induced T cell apoptosis were examined. Our results demonstrate that ARD-induced T cell apoptosis was not dependent on Fas/Fas ligand (FasL) interaction. Interestingly, ARD-induced DNA damage and phosphatidylserine (PS) externalization were independently regulated, and selective prevention of DNA damage by caspase inhibitors could not rescue T cells from death. In addition we show that ARD-induced T cell DNA damage appeared to require different caspase activities. The selective toxicity toward T cell lineages, especially toward activated T cells, by Tet provided strong support for further investigation of this drug in the therapy of autoimmune diseases.

Materials and Methods

Preparation of ARDs

HCQ was obtained as tablets or as a purified compound from Sanofi-Winthrop Pharmaceuticals (New York, NY). Drugs were dissolved in H2O to make a 30 mg/ml stock solution. Results obtained using HCQ tablets were confirmed with purified compounds. The two preparations did not show significant differences in effects in our studies. MTX was purchased from Calbiochem (La Jolla, CA), and dissolved in H2O. The stock concentration was 50 mM. The powder of Tet, C38H42O6N2, with a purity of >98% was obtained from Yichang Pharmaceutical Co. (Hubei Province, Yichang, People’s Republic of China) and dissolved in 0.1 N HCl. The stock concentration was 5 mM (15). Tablets of TWH containing 33 μg of ethyl acetate extract were purchased from Huanshi Pharmaceutical Co. (Huishan, Hubei, People’s Republic of China). The tablets were ground into powder, dissolved in 10% DMSO and 20% ethanol, and then filtered through a 0.45-μm pore size filter paper. The stock concentration was 10 μg/ml. For the requirements of each experiment, each drug was made by further dilution of the concentrated stock solution with culture medium. The final concentrations of HCl, DMSO, and ethanol in the experiments did not have any effect on cell viability.

Cells, reagents, Abs, and cell stimulation

Human T cell lines, Jurkat, Molt-4, and Sup-T1 and the monocytic cell line, U937, were purchased from American Type Culture Collection (Manassas, VA). The human B cell line, Ramos, and another human T cell line, CEM, were provided by S. L. Hsieh (National Yang-Ming University, Taiwan). The other monocytic cell line, THP-1, was a gift from L. Y. Chau (Academia Sinica, Taiwan). The cell lines were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, and 100 U/ml of penicillin-streptomycin (Life Technologies, Gaithersburg, MD). Human peripheral blood T cells were purified from whole blood by negative selection on magnetic-activated cell sorting (MACS) (28). In brief, whole blood (20–50 ml) from healthy donors (>70 participants) or buffy coat were treated with drugs immediately after lysis of RBC, the PBMC were placed on petri dishes to remove adherent cells and incubated with Abs, including L243 (anti-DR; American Type Culture Collection), OKM1 (anti-CD11b; American Type Culture Collection), and L243 (anti-CD11b; American Type Culture Collection) for 30 min at 4°C. The cells were then washed with medium containing 10% FBS and incubated with magnetic beads conjugated with goat anti-mouse IgG (R&D Systems, Minneapolis, MN). The Ab-stained cells were removed with a magnet. Following a repeat of the above procedures, the purity of the T cells was shown to be >98% as determined by the percentage of CD3+ cells in a flow cytometer (Becton Dickinson, Mountain View, CA). For the stimulation experiments, PMA (Sigma, St. Louis, MO) at 5 ng/ml and ionomycin (Sigma) at 1 μM were used. All caspase inhibitors were purchased from Calbiochem. All reagents for flow cytometry analysis were purchased from PharMingen (San Diego, CA) or Becton Dickinson. Unless otherwise indicated, the rest of the reagents were purchased from Sigma.

Preparation and treatment of resting and activated T cells from same donors

To compare the cytotoxicity of ARDs on resting and activated T cells, purified T cells from buffy coat were treated with drugs immediately after preparation as described or were activated with IL-2 at 10 IU/ml for 3 days. After stimulation with IL-2, the cell debris and dead cells were removed by Ficoll-Hypaque density gradient centrifugation. The activated T cells were then treated with ARDs in the absence of exogenous IL-2 as described for the resting T cells.

Measurement of nonspecific cytotoxicity

Several assays were used to examine drug toxicity in human peripheral blood T cells and different human cell lines. The release of lactate dehydrogenase (LDH) as an indicator of damage to the plasma membrane and cell death was measured according to the manufacturer’s instructions (Roche, Indianapolis, IN). The percent cytotoxicity was calculated as (sample value – medium control) ÷ (high control – medium control) × 100, where the sample value is the average of absorbance values of the triplicates of drug-treated cell supernatants after subtraction from each of the absorbance values obtained in the background control. Similarly, the average absorbance values of untreated cell supernatants, used as the medium control, were calculated. Equal amounts of cells treated with 1% Triton X-100 were used as the high control. Trypan blue exclusion assays and MTT assays were performed as described in our previous work (26).

Measurement of cellular apoptosis by flow cytometry analysis

After washing with cold PBS, the drug-treated and untreated cells were pelleted and resuspended in binding buffer containing HEPES-buffered PBS supplemented with 2.5 mMol CaCl2. Then 10 μl of annexin V-FITC (10 μg/ml) and 10 μl of propidium iodide (PI; 50 μg/ml) were added to each sample and incubated for 15 min at room temperature. After washing, the cells were analyzed by flow cytometry (Becton Dickinson). The annexin V+ and PI− population represents an early apoptotic population of cells, and the late apoptotic or necrotic population was annexin V+ and PI+. Where PI staining was measured alone, the cells were pelleted and resuspended in 1.5 ml of hypotonic fluorochrome solution containing 50 μg/ml of PI, 0.1% sodium citrate, and 0.1% Triton X-100 (Sigma). The mixture was left at 4°C in the dark overnight. While cells were not permeabilized in simultaneous staining with annexin V-FITC and PI, Triton X-100 was added to permeabilize cells stained only with PI. The PI fluorescence intensity was then measured with a flow cytometer, and the sub-diploid DNA content was analyzed with the CellQuest program (Becton Dickinson).

Measurement of cell surface molecule expression

For analysis of the expression of Fas and FasL, the untreated and drug-treated cells were stained with FITC-conjugated anti-Fas mAb or mouse anti-FasL mAb and then with FITC-conjugated goat anti-mouse mAb (PharMingen). To prevent shedding of FasL, 30 min before treatment a matrix metalloprotease inhibitor (Ebs301, PharMingen; 10 μM) was added to the culture. The expression of Fas/FasL molecules was measured by flow cytometry (Becton Dickinson). The FITC-conjugated isotype-matched mAbs were used as controls.

Measurement of DNA fragmentation

T cells were washed, pelleted, and resuspended in 100 μl (for 4 × 106 cells) of hypotonic lysis buffer (1% Triton X-100, 50 mM Tris-HCl (pH 7.9), 10 mM EDTA, and 50 μg/ml RNase A) for 10 min at room temperature. After centrifugation at 16,100 × g for 20 min, the supernatant was put through a DNA Miniprep procedure (Promega, Madison, WI). Then sequential washes with 750 and 250 μl of 70% ethanol were performed, and the DNA was eluted with 100 μl of water at 65°C and concentrated to ~20 μl in a Speed-Vac. The DNA was subsequently analyzed on a 1 or 2% agarose gel in 0.5× TAE buffer (40 mM Tris base, 2 mM EDTA, and 20 mM glacial acetic acid), stained with ethidium bromide, and detected under UV light.

Generation of Fas-sensitive and Fas-resistant Jurkat T cells

The parental human leukemic T cell line Jurkat was used as the Fas-sensitive cell line. A Fas-resistant cell line was generated by continual growth of Jurkat T cells in the presence of 50 ng/ml of anti-Fas IgM mAb (Upstate Biotechnology, Lake Placid, NY). The dead cells were removed by Ficoll-Hypaque density gradient centrifugation before each change of the medium. After culture in this medium for 3 mo, Jurkat T cells became fully Fas resistant, as indicated by the absence of Ab-induced cell death in trypan blue exclusion assays. The Fas-resistant Jurkat T cells, after culture for 3–9 mo, were used in this study.

Western blotting

ECL Western blotting (Amersham, Arlington Heights, IL) was performed as we previously described (29). Briefly, after extensive washing, the
treated and untreated cells were pelleted and resuspended in lysis buffer (20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25% glycerol, 1 mM DTT, 1 mM PMSF, and 3.3 μM of aprotinin/ml). After periodic vortexing for 1 h, the mixture was centrifuged at 16,100 × g for 20 min, the supernatant was collected, and the protein concentration was measured by Lowry assay. Equal amounts (100 μg) of whole cellular extracts were analyzed on 15% SDS-PAGE. The protein was then transferred to a nitrocellulose filter. For immunoblotting, the nitrocellulose filter was incubated with TBS-Tween 20 containing 5% nonfat milk (milk buffer) for 2 h, and then blotted with antisera against β-actin (Chemicon, Temecula, CA), Fas-associated death domain protein (FADD), caspase-8, or caspase-3 (PharMingen) overnight at 4°C. After washing with milk buffer twice for 20 min each time, the filter was incubated with donkey anti-mouse IgG conjugated to HRP at a concentration of 1/5000 for 30 min. The filter was then incubated with the substrate for 1 min and exposed to x-ray film.

Measurement of caspase-3 activity

This procedure was performed according to the manufacturer’s instructions (Clontech, Palo Alto, CA). In brief, after washing, the untreated and treated cells were pelleted and fixed with 1% paraformaldehyde overnight. Following washing with cold PBS, cells were permeabilized by adding 0.25% saponin, and then anti-caspase-3 mAb conjugated with PE was added and incubated for 30 min at 4°C in the dark. After washing, the active caspase-3 product was analyzed in a flow cytometer (Becton Dickinson).

Measurement of caspase-8 activity

The measurement of caspase-8 activity was performed according to the manufacturer’s instructions (Clontech). T cells (8 × 10⁶) were lysed, and after centrifugation at 16,100 × g for 20 min, the supernatants were collected, and the protein concentrations were measured by Lowry assay. Two hundred micrograms of protein in a 50-μl volume were mixed with an equal volume of 2× reaction buffer and 5 μl of 4 mM IETD-pNA substrate. The mixture was incubated at 37°C for 2 h and then read at 405 nm in a microplate reader (Dynatech, Chantilly, VA).

Statistics

When necessary, the results were expressed as the mean ± SD. Unpaired Student’s t test was used to analyze the data; p < 0.05 was considered significant.

Results

Effect of ARDs on human peripheral blood T cells

When MTX is administered in weekly doses (7.5–20 mg/wk, in three divided doses taken every 12 h), its serum concentration is maintained at 1 nM (30). However, much higher concentrations (the average maximum concentration is 700 nM) can be achieved temporarily after an oral dose of 15 mg (30, 31). The serum concentration quickly drops from 700 to ~100 nM within 12 h and then reaches 1 nM after administration of the medication (30). It has been shown that serious toxicity is likely if a serum concentration of 300 nM or more is maintained ~36 h (32). In the present study because the cytotoxicity of ARDs was examined after 12-, 24-, and 48-h incubation with drugs, a concentration of 100 nM MTX was chosen to evaluate its cytotoxicity on T cells. Similarly, the optimal antirheumatic concentrations of HCQ, Tet, and TWHf were examined for their effects on purified human peripheral blood T cells (13, 14, 21, 33). The structures of MTX, HCQ, and Tet are shown in Fig. 1A. Several approaches, including trypan blue exclusion assays (Fig. 1B), staining with PI (Fig. 1C), and measurement of LDH release (Fig. 1D) were used to examine...
drug cytotoxicity. We show that three ARDs, HCQ, TWHf, and Tet, but not MTX, at antirheumatic concentrations, could induce T cell death (Fig. 1, B–D). As indicated, because the concentration of 100 nM MTX could be maintained in serum for <12 h after taking 15 mg orally, and treatment with this concentration for 48 h did not affect T cell survival, this drug was no longer examined in our subsequent studies. HCQ was less toxic to T cells than the other two ARDs.

Activated T cells were more susceptible to Tet-induced cytotoxicity

The effects of ARDs on resting and IL-2-activated T cells were compared. Human peripheral blood T cells from the same donors were either treated with ARDs immediately after preparation or stimulated with IL-2 at 10 IU/ml for 3 days. After removal of dead cells, the IL-2-activated T cells were similarly treated with ARDs. Although treatment for 24 h with either HCQ or TWHf induced stronger cytotoxicity on activated than on resting T cells, as assayed by trypan blue exclusion (Fig. 2A), these results were not supported by the other two cytotoxicity assays (Fig. 2, B and C). In contrast, we found that IL-2 stimulation rendered T cells more susceptible to Tet-induced cytotoxicity. This conclusion was supported by three different cytotoxicity measurements (Fig. 2, A–C).

Tet-induced cell death appeared to be highly specific toward T cells

We next investigated whether ARD-induced cytotoxicity is specific to T cells. The cytotoxic effects of ARDs on several human immune effector cell lines were examined. Fig. 3 shows that compared with B and monocytic cell lines, T cells were much more susceptible to Tet-induced cytotoxicity. Among the T cell lines examined, Jurkat and Molt-4 were the most sensitive, as analyzed by cell viability (Fig. 3A) and LDH release (Fig. 3B). Interestingly, we found that another human T cell line, HuT-78, was totally resistant to Tet-mediated cytotoxicity (unpublished observations). The HuT-78 T cell line has many characteristics that differ from those of other commonly used T cell lines, such as much lower expression of CD2, CD4, and CD45 as well as much higher resistance to anti-Fas- and TNF-α-mediated cell death (34, 35). The signaling pathway that mediates Tet-induced cytotoxicity may be altered in this particular cell line. Although TWHf showed some preferential cytotoxicity to T cells, the differences were less marked than with Tet (Fig. 3, A and B). Under the same conditions, the cytotoxic effects of HCQ varied according to the assays performed (Fig. 3, A and B).

ARDs mediated cytotoxicity through apoptosis

The mechanisms of the cytotoxic effects of ARDs were examined. For this purpose, we used 2-fold higher concentrations of the drugs to treat cells. This concentration of HCQ can be obtained in vivo, and its therapeutic effects were greater during a short-term clinical investigation (36). After treatment for 3 h, all ARDs increased the percentages of T cells that stained positively for annexin V-FITC (an early apoptotic sign) and PI (a late apoptotic or necrotic sign). Compared with 3-h treatment, T cells treated with ARDs for 8 h...
produced apoptosis was further examined in both Fas-sensitive and Fas-resistant Jurkat T cells.

The lack of a requirement for Fas/FasL interaction in ARD-induced apoptosis in Fas-sensitive and Fas-resistant Jurkat cells was also demonstrated by DNA fragmentation assay (Fig. 5A). As shown in Fig. 5A, both Fas-sensitive and Fas-resistant Jurkat T cells were equally susceptible to apoptosis induced by the three ARDs (Fig. 5A). These results demonstrated that the mechanism of T cell apoptosis induced by ARD treatment might not involve Fas/FasL interaction.

ARDs did not induce Fas/FasL expression

Because Fas/FasL interaction is crucial in some forms of T cell apoptosis, we then determined whether ARD-induced T cell apoptosis is dependent on Fas/FasL interaction. As shown in Fig. 5A, ARD treatment did not increase Fas expression in kinetic studies. Under identical conditions, ARDs also failed to induce FasL expression (unpublished observations). In contrast, similar to other reports, PMA plus ionomycin stimulation caused apparent induction of Fas expression on T cells (Fig. 5B). Because both FADD and caspase-8 were shown to be essential in Fas-mediated apoptosis (13–14), the expression of these two molecules was also examined was not markedly affected (Fig. 6A). Because both FADD and caspase-8 were shown to be essential in Fas-mediated apoptosis (37–39), the expression of these two molecules was also examined by Western blotting. Fig. 6C shows that the levels of expression of both FADD and caspase-8 in these cell lines were similar or identical. Thus, the only detectable difference between Fas-sensitive and Fas-resistant Jurkat T cells was the reduced number of Fas molecules expressed on Fas-resistant cells (Fig. 6B). As shown in Fig. 6D, compared with Fas-sensitive Jurkat T cells, Fas-resistant T cells were less susceptible to anti-CD3-induced apoptosis. Under the same conditions, both Fas-sensitive and Fas-resistant cells were equally susceptible to apoptosis induced by the three ARDs (Fig. 6D). These results demonstrated that the mechanism of T cell apoptosis induced by ARD treatment might not involve Fas/FasL interaction.

Effect of caspase inhibitors on ARD-induced apoptosis in human peripheral blood T cells

Caspase enzymes are known to be involved in the signaling pathway of apoptosis (40). The mechanisms of ARD-induced T cell apoptosis were analyzed using caspase inhibitors. As illustrated, both Z-Val-Ala-Asp-fluomethyl ketone (Z-VAD-fmk), a generalized caspase inhibitor, and Z-Asp-Glu-Val-Asp-fluomethyl ketone (Z-DEVD-fmk), a caspase inhibitor that targets mainly caspase-3, but also other inhibitors, could induce strong DNA fragmentation, although the kinetics were different. These observations were consistent with morphological changes in ARD-induced T cell apoptosis (13, 14).

ARD-induced apoptosis in Fas-sensitive and Fas-resistant Jurkat T cells

The lack of a requirement for Fas/FasL interaction in ARD-induced apoptosis was further examined in both Fas-sensitive and Fas-resistant Jurkat T cells (Fig. 6A). The generation of a Fas-resistant cell line resulted in the reduction of Fas expression on cell surface, but the expression of other T cell surface molecules examined was not markedly affected (Fig. 6B). Because both FADD and caspase-8 were shown to be essential in Fas-mediated apoptosis (37–39), the expression of these two molecules was also examined by Western blotting. Fig. 6C shows that the levels of expression of both FADD and caspase-8 in these cell lines were similar or identical. Thus, the only detectable difference between Fas-sensitive and Fas-resistant Jurkat T cells was the reduced number of Fas molecules expressed on Fas-resistant cells (Fig. 6B). As shown in Fig. 6D, compared with Fas-sensitive Jurkat T cells, Fas-resistant T cells were less susceptible to anti-CD3-induced apoptosis. Under the same conditions, both Fas-sensitive and Fas-resistant cells were equally susceptible to apoptosis induced by the three ARDs (Fig. 6D). These results demonstrated that the mechanism of T cell apoptosis induced by ARD treatment might not involve Fas/FasL interaction.

Fas-resistant Jurkat T cells (Fig. 6A). The generation of a Fas-resistant cell line resulted in the reduction of Fas expression on cell surface, but the expression of other T cell surface molecules examined was not markedly affected (Fig. 6B). Because both FADD and caspase-8 were shown to be essential in Fas-mediated apoptosis (37–39), the expression of these two molecules was also examined by Western blotting. Fig. 6C shows that the levels of expression of both FADD and caspase-8 in these cell lines were similar or identical. Thus, the only detectable difference between Fas-sensitive and Fas-resistant Jurkat T cells was the reduced number of Fas molecules expressed on Fas-resistant cells (Fig. 6B). As shown in Fig. 6D, compared with Fas-sensitive Jurkat T cells, Fas-resistant T cells were less susceptible to anti-CD3-induced apoptosis. Under the same conditions, both Fas-sensitive and Fas-resistant cells were equally susceptible to apoptosis induced by the three ARDs (Fig. 6D). These results demonstrated that the mechanism of T cell apoptosis induced by ARD treatment might not involve Fas/FasL interaction.
caspases, such as caspase-6, -7, -8, and -10, effectively reduced the ARD-induced subdiploid DNA content (Fig. 7A), but had no effect on inhibition of PS externalization (Fig. 7B). To further examine the extent of DNA damage, DNA was extracted from ARD-treated cells in the presence or the absence of caspase inhibitor pretreatment and analyzed in agarose gels. Consistently, both caspase inhibitors could inhibit ARD-induced DNA fragmentation (Fig. 7C). We also found that the susceptibility of T cells to caspase inhibitors was similar at different time points (8, 12, and 24 h) of ARD treatment (our unpublished observations). The susceptibilities of DNA damage and PS externalization to caspase inhibitors were further investigated with both trypan blue exclusion assays and LDH release. We found that the prevention of DNA damage by caspase inhibitors still rendered T cells permeable to trypan blue (Fig. 7D) as well as the release of LDH (Fig. 7E). These observations suggest that the processes of DNA damage and PS externalization can be independent, and that the triggering of either process can lead to T cell death. In this context, ARD-induced T cell apoptosis was mediated through both caspase-dependent and caspase-independent signaling pathways, and caspase activities were involved only in ARD-induced T cell DNA damage. In addition, we found that none of these effects of ARD-induced T cell apoptosis could be affected by the caspase-1 inhibitor, Ac-YVAD-cho (unpublished observations). Because we could not detect any inhibitory effect of this caspase inhibitor on T cell apoptosis induced by anti-Fas, it is difficult to draw any conclusion about the role of caspase-1 in ARD-induced T cell apoptosis.

Activation of caspase-3 in Tet- and TWHf-treated, but not HCQ-treated, Fas-sensitive and Fas-resistant Jurkat T cells

Although Z-DEVD-fmk primarily inhibits caspase-3 activity, it may also target other caspase enzymes (41). The role of caspase-3 in ARD-induced T cell DNA damage was further examined with immunoblotting assays in Fas-sensitive and Fas-resistant Jurkat T cells. As shown in Fig. 8A, Tet-treatment induced the cleavage of procaspase-3 and led to the generation of processed forms. Because this finding was observed in both Fas-resistant (Fig. 8A) and Fas-sensitive (Fig. 8B) Jurkat T cells, it indicates that Tet-induced caspase-3 activation bypasses Fas molecules and, therefore, is independent of Fas/FasL interaction. Consistently, although Z-DEVD-fmk could block TWHf-induced DNA fragmentation only at the highest concentration, TWHf significantly induced the processing of caspase-3 in both Fas-resistant (Fig. 8C) and Fas-sensitive (Fig. 8D) Jurkat T cells. Unexpectedly, under the same conditions HCQ at an even higher concentration (60 μg/ml) did not induce more processed forms of caspase-3 (Fig. 8E and F).

Detection of an increased cell population expressing active form of caspase-3 in TWHf- and Tet-treated, but not HCQ-treated, human peripheral blood T cells

To analyze the activation of caspase-3 by ARDs in human peripheral blood T cells, the intracellular protein levels of active caspase-3 were measured with a flow cytometer. As shown in Fig. 9, both Tet and TWHf, but not HCQ, significantly increased the percentage of cells expressing the active caspase-3 product. Consistent with the Western blotting results, HCQ at 60 μg/ml did not activate caspase-3 as determined by flow cytometry analysis of human peripheral blood T cells (our unpublished observations).

HCQ failed to induce caspase-8 activity

Because Z-DEVD-fmk could block HCQ-induced T cell DNA damage in which caspase-3 was not involved, we examined whether caspase-8 could be the target of Z-DEVD-fmk. As shown in Fig. 10, in contrast to anti-Fas IgM mAb stimulation, HCQ treatment did not induce caspase-8 activity. Thus, although HCQ-induced T cell DNA damage was sensitive to Z-DEVD-fmk, neither caspase-3 nor caspase-8 appeared to participate in this apoptotic signaling pathway.

Discussion

Apoptosis is an important part of the process of embryogenesis, for deletion of strongly autoreactive T cells in the thymus, and to protect immunologically privileged sites from autoimmunity (42, 43). In contrast to the necrotic process, the apoptotic process does not induce an inflammatory response because dead cells are rapidly phagocytosed before leakage of cellular contents (42). Although the etiology of autoimmune diseases is largely unknown, the immune reaction directed against apoptotic bodies or Ags expressed from dead cells has been implicated as one mechanism that leads to autoimmune diseases (44–47). After the development of an autoimmune disease, defective execution of the apoptotic program may sustain the inflammatory process (8, 48–53). Therefore, the development or identification of drugs that induce apoptosis in activated T cells or increase the susceptibility to apoptosis would be a useful approach in the treatment of autoimmune diseases (11).

In the present study we show that several ARDs, HCQ, TWHf, and Tet, can cause T cell apoptosis at therapeutic antirheumatic
concentrations (Figs. 1 and 4). Further investigation of the apoptotic mechanism revealed that Fas/FasL interaction was not involved in ARD-mediated T cell apoptosis (Figs. 5 and 6). Interestingly, during the ARD-induced T cell apoptotic process, externalization of PS, which is considered to be an early indication of apoptosis, and DNA damage, a late apoptotic sign, were differentially regulated. Inhibition of caspase activity blocked ARD-induced DNA damage, but not ARD-induced PS externalization (Fig. 7, A–C). In addition, prevention of DNA damage by caspase inhibitors could not rescue T cells from ARD-induced cell death (Fig. 7, D and E). When the role of different caspases was examined, we found that caspase-3 played an important role in both TWHf- and Tet-induced T cell DNA damage. However, HCQ-induced T cell DNA damage used Z-DEVD-fmk-sensitive caspase cascades, in which caspase-3 and caspase-8 were not involved (Figs. 8–10). These observations are summarized in Fig. 11. Because the most effective therapy for autoimmune diseases at present uses a combination of several DMARDs with different mechanisms, the differential use of caspase activities by ARDs may have implications for future use of a combination of both Western and Chinese ARDs in the therapy of these illnesses. Although there appears to be interesting biology associated with both HCQ and TWHf, the lack of T cell specificity of these two drugs does raise a concern about their mechanism of action. In this context, Tet showed great selectivity toward T cell lineages, especially toward IL-2-activated T cells (Fig. 2).

In contrast to apoptosis induced by some anticancer drugs as well as other stimuli, such as UV irradiation, anti-CD3 stimulation, and mitogen treatment (54–59), Tet induced T cell apoptosis through a mechanism independent of Fas/FasL interaction. This conclusion was supported by several observations. First, the expression of Fas/FasL mRNA was not enhanced by Tet treatment (our unpublished observations). Second, Tet did not increase Fas/FasL expression on the T cell surface (Fig. 5). Third, both Fas-sensitive and Fas-resistant T cells showed comparable susceptibility to Tet-induced apoptosis (Fig. 6). Lastly, Tet-induced activation of caspase-3 appeared to have similar kinetics and intensity in Fas-sensitive and Fas-resistant Jurkat T cells to ARD-induced apoptosis. Both Fas-sensitive and Fas-resistant Jurkat T cells were treated with immobilized anti-CD3 (10 μg/ml), HCQ (30 μg/ml), TWHf (30 ng/ml), or Tet (30 μM) for various time periods, and cell survival was examined with MTT assays. Data shown are the mean of the triplicate determinations for each treatment. This experiment was performed at least three times with similar results.

**FIGURE 6.** ARD-induced apoptosis in Fas-sensitive and Fas-resistant Jurkat T cells. A, Susceptibility of Fas-sensitive and Fas-resistant Jurkat T cells to treatment with various doses of anti-Fas IgM mAb. Both Fas-sensitive and Fas-resistant Jurkat T cells were treated with 50, 75, or 100 ng/ml of anti-Fas IgM mAb for 24 h, and cell survival was examined by MTT assays. B, Expression of cell surface molecules on Fas-sensitive and Fas-resistant Jurkat T cells. After staining of Fas-sensitive and Fas-resistant Jurkat T cells with various mAbs against cell surface molecules as indicated, the expression of these molecules was examined with a flow cytometer. C, Expression of β-actin, FADD, and caspase-8 in both Fas-sensitive (Fas-S) and Fas-resistant (Fas-R) Jurkat T cells was examined by Western blotting, as described in Materials and Methods. D, Similar susceptibility of Fas-sensitive and Fas-resistant Jurkat T cells to ARD-induced apoptosis. Both Fas-sensitive and Fas-resistant Jurkat T cells were treated with immobilized anti-CD3 (10 μg/ml), HCQ (30 μg/ml), TWHf (30 ng/ml), or Tet (30 μM) for various time periods, and cell survival was examined with MTT assays. Data shown are the mean of the triplicate determinations for each treatment. This experiment was performed at least three times with similar results.
We were surprised to observe that Tet-induced DNA damage and PS externalization had differential susceptibility to caspase inhibition. In this context there are several recent reports concerning the existence of caspase-independent apoptotic mechanisms (66-71). Similar to Tet, some stimuli elicit both caspase-dependent and caspase-independent apoptotic effects (72, 73) (Fig. 11). Regarding the manifestations of apoptosis, PS externalization may be regulated independently from caspase activation (74, 75), and its appearance has been reported in thymocyte necrosis (76), calcium-triggered T cell necrosis (77), and serum amine oxidase-induced necrosis in mouse leukemic cells (78). Although externalization of PS is considered to be an important marker for phagocytes to engulf apoptotic cells, necrotic cells could still be recognized and phagocytosed by macrophages in the absence of PS externalization (77). Furthermore, in a model of a differentiation stimulus hemin-induced apoptosis in erythroleukemic cells, the caspase inhibitor, Z-VAD-fmk, could block hemin-induced DNA fragmentation, but it could not down-regulate hemin-induced PS externalization (79). This observation is similar to our findings. Compared with PS externalization, fragmentation of DNA occurs later in apoptosis and may not occur in apoptosis induced by some stimuli (48, 80, 81), but DNA fragmentation resulting in 180- to

FIGURE 7. Effect of caspase inhibitors on ARD-induced T cell apoptosis. Human peripheral blood T cells were pretreated with Z-VAD-fmk (100 μM in A, B, D, and E and three doses in C as indicated) or with Z-DEVD-fmk (100 μM in A, B, D, and E and three doses in C as indicated) for 3 h. The cells were then treated with HCQ (30 μg/ml), TWHf (30 ng/ml), or Tet (30 μM) for another 24 h (A, B, D, and E) or variable time points (C; 12 h for Tet, 24 h for HCQ, and 20 h for TWHf). The apoptotic effects were examined by staining with PI (A), annexin V-FITC (B), DNA fragmentation assays (C), trypan blue exclusion assays (D), and LDH release measurements (E) as described in Materials and Methods. The percentages of PI+ and annexin V+ T cells in medium control at the 24 h point were 5 and 11%, respectively. C, * represents 100-bp DNA ladder markers (100–1000 bp). The concentrations of agarose gels used for analysis are 1% (for Tet-treated cells) and 2% (for TWHf- and HCQ-treated cells). The representative data shown were obtained from >10 different donor T cells with similar results.
200-bp DNA ladders is found only in cells undergoing apoptosis and not in those undergoing necrosis. Because it has been reported that the apoptotic process can be switched to a necrotic death (82–84) in the presence of caspase inhibitors, we could not exclude the possibility that this might happen in our system, especially when cell survival was evaluated based upon trypan blue exclusion and LDH release. In the presence of caspase inhibitors, determination of whether the Tet-treated cells are dying from apoptosis, necrosis, or oncosis (85, 86) requires further analysis, including the evaluation of other morphological, biochemical, and molecular characteristics of these three types of cell death. Moreover, our observations raise a serious concern about which assays should be used to examine models of apoptosis.

**FIGURE 8.** Detection of caspase-3 activation in Tet- and TWHf-treated, but not HCQ-treated, Fas-sensitive and Fas-resistant Jurkat T cells by Western blotting. Fas-resistant (A) or Fas-sensitive (B) Jurkat T cells were treated without or with Tet at 30 μM for various time periods. Similarly, Fas-resistant (C) and Fas-sensitive (D) Jurkat T cells were treated without or with TWHf at 30 ng/ml for the indicated time periods. Fas-sensitive Jurkat T cells treated without or with HCQ at 30 μg/ml (E) or 60 μg/ml (F) were also analyzed. For each treatment the vehicle-treated cells were included as a control. Equal amounts of proteins (100 μg) were run through 15% SDS-PAGE, transferred to nitrocellulose papers, and probed with anti-caspase-3 mAb as described in Materials and Methods. The procaspase-3 (+) and its processed forms (++) were indicated. F-R, Fas-resistant Jurkat T cells.

**FIGURE 9.** Induction of active caspase-3 product in Tet- and TWHf-treated, but not HCQ-treated, human peripheral blood T cells. T cells treated with vehicle (indicated as –Ctl), HCQ (30 μg/ml), TWHf (30 ng/ml), or Tet (30 μM) for various time periods were collected, and the intracellular protein levels of active caspase-3 product were determined with a flow cytometer. Data shown are representative results from four individual experiments. The control shown for TWHf- and HCQ-treated cells includes only the 24 h point.

**FIGURE 10.** Caspase-8 was not required for HCQ-induced T cell apoptosis. Jurkat T cells untreated or treated for various periods with vehicle (Med), HCQ at 30 μg/ml, or anti-Fas IgM mAb at 100 ng/ml were assayed for caspase-8 activity as described in Materials and Methods. Data shown are representative results from four individual experiments.
It is noteworthy that the apoptotic effect of Tet appeared to be highly selective toward T cell lineages, especially activated T cells (Figs. 2 and 3). According to Li et al. (21), the administration of Tet to a 15-year-old patient with a 15-yr history of RA led to a discontinuance of the medication (21). In our in vitro system, incubation with 15-μM (9.3 μg/ml) concentration of Tet for 48 h was enough to kill >80% of T cells. This evidence argues against a general toxicity of Tet toward all tissue cells. Although the T cell counts were not determined, Li et al. (21) did detect a reduction of serum IgG in Tet-treated silicosis patients. This finding suggests an inhibition of T or B cell activation in these Tet-treated patients. Because T cells play a critical role in the regulation of immune responses, any agent that effectively and selectively inhibits T cell activation and/or induces T cell apoptosis should have great potential in the therapy of autoimmune diseases and in the prevention of transplantation rejection.

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