Type of Cell Death Induced by α-Trifluoromethyl Acyloins in Oral Squamous Cell Carcinoma

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Abstract. *We previously reported that α-trifluoromethyl acyloins (TFs) induced various types of cell death, depending on the target cancer cell line. We investigated here what type of cell death is induced by α-trifluoromethyl acyloins in two human oral squamous cell carcinoma cell lines (HSC-2, HSC-4). TFs produced few TUNEL-positive cells. TFs induced annexin V/PI-double positive HSC-2 cells and annexin V-positive/PI-negative HSC-4 cells, respectively, but failed to activate caspase-3, capase-8 and caspase-9 in both HSC-2 and HSC-4 cells. On the other hand, TFs induced the formation of acidic organelles (detected by acridine orange staining) in both HSC-2 and HSC-4 cells. When HSC-2 and HSC-4 cells that had been transfected with expression vector encording the microtubule-associated protein 1 light chain 3 (LC3) gene fused to green fluorescent protein (GFP) were treated with TFs, LC3-GFP fusion protein was accumulated as granular dots in autophagosomes. Pretreatment with 3-methyladenine, an inhibitor of autophagy, partially inhibited the cytotoxicity of TFs, the formation of acidic organelles and LC3 accumulation in the autophagosome. These data suggest that α-trifluoromethyl acyloins may induce autophagic cell death in HSC-2 and HSC-4 cells following the early stage of necrosis or apoptosis, respectively.*

Trifluoromethyl ketones (TFs) have been shown to be inhibitors of serine and cysteine proteases (1). The strong electron-withdrawing character of the trifluoromethyl group alters the properties of the carbonyl group and increases electrophilicity (2). Recent efforts have been devoted to the discovery of new biological activities such as anti-*Helicobacter pylori* (3), antibacterial (4), SARS-CoV 3CL protease inhibitory (5) and histone deacetylase inhibitory (6) activities. We have recently reported the tumor-specific cytotoxicity of six structurally-related TF compounds **(TF1-6),** based on the tumor-specificity index (TS) defined as the ratio of the mean 50% cytotoxic concentration (CC_{50}) value against three normal human cells [gingival fibroblasts (HGF), pulp cells (HPC), periodontal ligament fibroblasts (HPLF)] to that for four human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, HSC-4 and promyelocytic leukemia HL-60) (7). α-Trifluoromethyl diketone [$CF_3COCOPh$ **(TF1)**] and two α-trifluoromethyl acyloins [$CF_3CH(OH)COPh$ **(TF2)**, $CF_3CH(OH)COCH_2Ph$ **(TF3)**] showed higher tumor-specificity (TS >3.5, 4.5 and 4.0, respectively) than the corresponding non-fluorinated analogs $CH_3COCOPh$ **(TF5)**, $CH_3CH(OH)COPh$ **(TF6)** (TS >1.0 and >1.0, respectively) and CF_3COCH_2Ph **(TF4)** (TS >1.6). The potency of cytotoxicity against four tumor cell lines was as follows: **TF2** (CC_{50}=21-119 μM) > **TF3** (33-108 μM) >**TF1** (30-223 μM)> **TF4** (90-279 μM)>> **TF5** (>239 μM) >**TF6** (>400 μM). These data suggest the importance of the α-hydroxy ketone moiety for cytotoxicity. **TF2** and **TF3** induced the formation of autophagosomes in HL-60 cells, without induction of any of apoptosis markers (internucleosomal DNA fragmentation, caspase-3 activity, production of apoptotic body, loss of cell surface microvilli) (7). However, **TF2** and **TF3** induced apoptotic cell death, characterized by DNA fragmentation (as identified by the TUNEL method) and activation of caspases (as identified by degradation products of cytokeratin 18 using M30 monoclonal antibody) in human submandibular gland carcinoma (HSG) cells (8). On the other hand, the structurally related α-trifluoromethyl diketone **(TF1)** (Figure 1) inhibited K^+ deprivation-induced apoptosis in cerebellar granule neurons (CGNs) (9, 10). These data suggest that α-trifluoromethyl acyloins **(TF2, TF3)** induce various types of cell death, depending on the target cancer cell line. In order to gain insight into the mechanism of action of these TFs, the type of cell death they induced by TFs in two human oral squamous cell carcinoma cell lines (HSC-2, HSC-4) was investigated.

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Materials and Methods

Chemicals. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chem Co., St Louis, MO, USA); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Osaka, Japan). **TF2** ($CF_3CH(OH)COPh$, MW=204) and **TF3** ($CF_3CH(OH)COCH_2Ph$, MW=218) (Figure 1) were prepared as described elsewhere (7), then dissolved in DMSO at 80 mM and added to the cell cultures at the indicated final concentrations. The final DMSO concentration in the culture medium was below 0.5% , a concentration that does not affect the growth of the cells.

Cell culture. HSC-2 and HSC-4 cells (provided by Professor Nagumo, Showa University) were cultured at 37˚C in DMEM supplemented with 10% heat-inactivated FBS in a 5% CO_2 incubator under a humidified atmosphere. Cells were washed with phosphate-buffered saline without Mg^{2+} and Ca^{2+} (PBS(–)), detached with 0.25% trypsin-0.025% EDTA-2NA in PBS(–) and subcultured twice a week.

Assay for the appearance of early marker of apoptosis. Cells (5×10^4/well) were plated in 8 well-chamber slides (BD Biosciences, CA, USA) and incubated for 48 hours. After the cells were treated for 12 hours with **TF2** or **TF3** (200 μM), they were washed with PBS(–) and resuspended in 85 μl of Binding Buffer (Medical Biology Laboratory, Nagano, Japan). The cells were added to 10 μl of annexin V-FITC and 5 μl of propidium iodide. After incubation at room temperature for 15 minutes in the dark, the cells were observed by a laser scanning microscope LSM510 (Carl Zeiss Inc., Gottingen, Germany), using the following filters: excitation 488 nm, emission 505-530 nm (green) and >580 nm (red) (11).

TUNEL assay. Cells (5×10^4/well) were inoculated on a 8-well chamber slide and incubated for 48 hours. Cells were treated for 12 hours with **TF2** or **TF3** (200 μM), and assayed for DNA cleavage by enzymatic end-labeling of DNA strand breaks, using a commercial apoptosis TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling of fragmented DNA) assay (Apo Alert DNA fragmentation assay kit; BD Biosciences). The treated cells were harvested and the cell pellet was suspended in 100 μl freshly prepared PBS(–) containing 4% formaldehyde, incubated at room temperature for 30 minutes, and centrifuged at 300 xg for 10 minutes. The pellet was washed once with 200 μl PBS(–) and suspended in 100 μl of PBS(–) containing 0.1% sodium citrate and 0.1% Triton® X-100, and stood for 2 minutes on ice. Cells were then washed twice with PBS(–). The pellet was resuspended in 50 μl TUNEL reaction mixture (45 μl labeling solution and 5 μl enzyme solution), incubated for 1 hour at 37°C in a humidified atmosphere in the dark and observed under laser scanning microscopy.

Assay for caspase activation. Cells were washed with PBS(–) and lysed in lysis buffer [50 mM Tris-HCl (pH7.5) 0.3% NP-40, 1 mM dithiothreitol]. After 10 minutes on ice and centrifugation for 5 minutes at 10,000 ×g, the supernatant was collected. Lysate (50 μl, equivalent to 200 μg protein) was mixed with 50 μl of the lysis buffer containing substrates for caspase-3 [DEVD-*p*-nitroanilide (*p*NA)], caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA) (Kamiya Biochem Co., Seattle, WA, USA). After incubation for 2 hours at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by a microplate reader (12, 13).

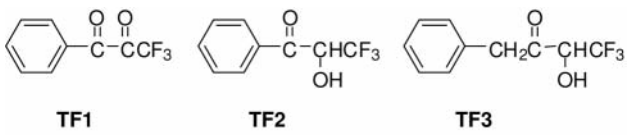

Figure 1. *Structures of **TF1**, **TF2** and **TF3**.*

Detection of acidic vesicular organelles with acridine orange staining. Acidic vesicular organelles were stained with acridine orange (Sigma Chemical Co.). Cells were stained with 0.1 μg/ml acridine orange for 20 minutes. Samples were then examined under laser scanning microscopy using the following filters: excitation 488 nm, emission 505-530 nm and >650 nm (14)

Assay for LC3 accumulation in the autophagosome. cDNA encoding LC3 was obtained by RT-PCR from the total RNA of HSC-2 cells with the LC3 sense primer (5'-GGGAATTCATGCCGTCGGAG AAGACCTT-3') and LC3 antisence primer (5'-GGGAATTC TAGATTACACTGACAATTTCATCC-3'). It was subcloned into the EcoRI site of pAcGFP1-C2, a GFP fusion protein expression vector (Clontech Laboratories, Inc., Mountain View, CA, USA). The plasmid constructs were verified by DNA sequencing using a Applied Biosystems 310 DNA sequencer (14)

HSC-2 or HSC-4 cells were seeded at 0.5×10^6 cells/well in 24-well plates, and the next day, the cells were transfected with a mixture of 1.5 μg of plasmid DNA and 0.7 μl of Lipofectamine™ 2000 (Invitrogen Corp., Carlsbad, CA, USA). After transfection for 18 hours, cells were treated for 4 hours with **TF2** or **TF3**. Mock transfection was performed using the empty pAcGFP1-C2 expression vector. GFP-LC3 transfected HSC-2 cells were observed by a laser scanning microscopy, using the following filters: excitation 488 nm, emission 505-530 nm, as described elsewhere (14).

Results

When HSC-2 cells were treated with **TF2** or **TF3**, some populations of TUNEL-positive cells with DNA cleaved by activated DNase(s) panel appeared (left, Figure 2). However, the percentage of TUNEL-positive cells was much lower than that induced by actinomycin D (positive control) (left panel, Figure 2). This indicates the weak DNA fragmenting activity of **TF2** and **TF3**. However, both **TF2** and **TF3** failed to produce any TUNEL-positive cells in HSC-4 cells (right panel, Figure 2).

TF2 and **TF3** also failed to activate caspase-3, caspase-8 and caspase-9 in both HSC-2 (Figure 3A) and HSC-4 (Figure 3B) cells during 4-24 hours treatment, whereas actinomycin D (positive control) significantly activated these caspases. The caspase activity rather declined at 24 hours after **TF** treatment, possibly due to the secondary result of cell death.

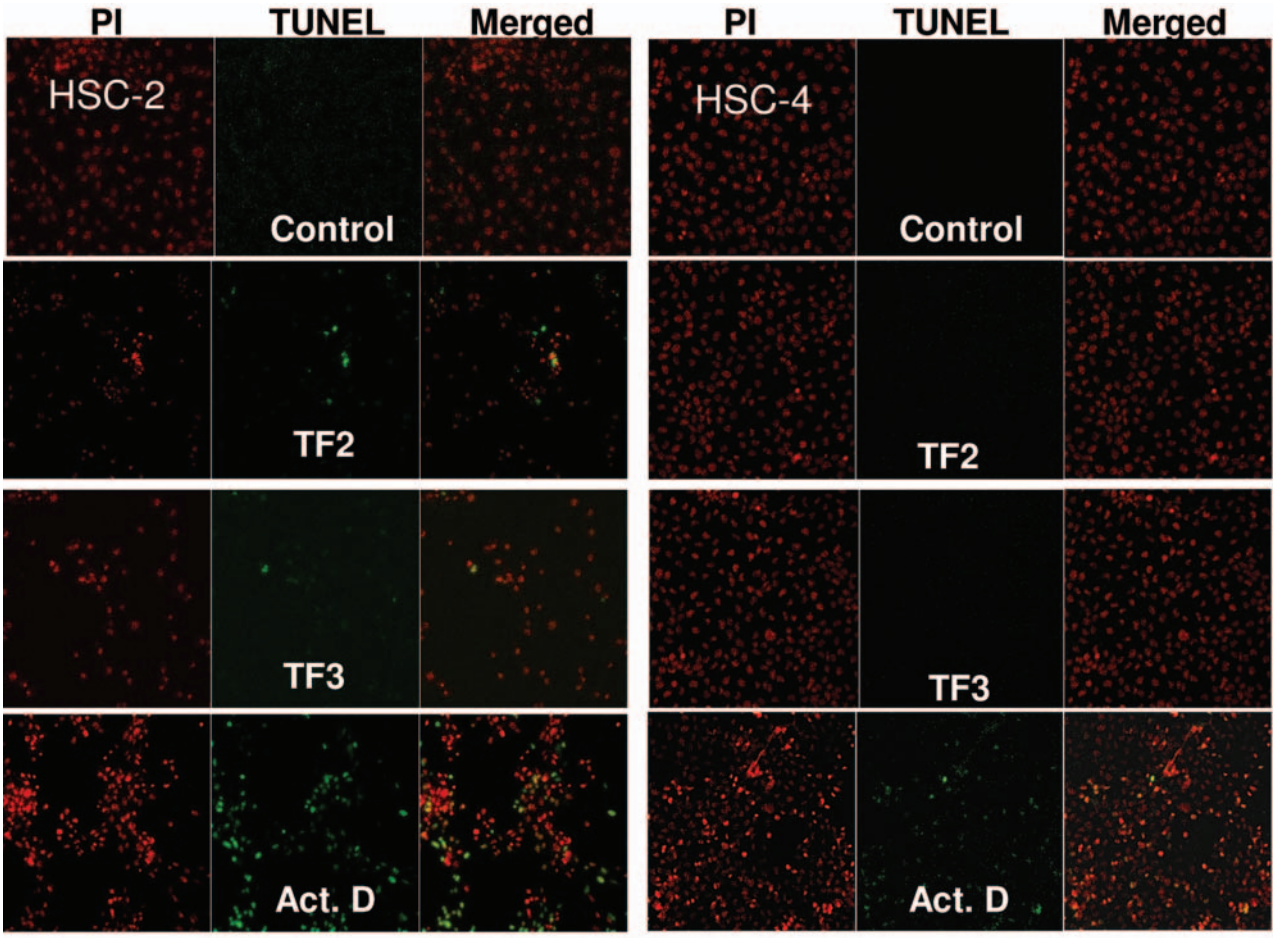

Figure 2. *Detection of DNA cleavage after treatment with **TF2** and **TF3**. HSC-2 (left panel) or HSC-4 cells (right panel) were seeded onto the surface of culture slides and cultured for 2 days until the cells reached a near-confluent monolayer. The cells were then treated for 12 hours with 200 μM **TF2**, 200 μM **TF3**, or 1 μg/ml actinomycin D (Act. D), and then subjected to the TUNEL assay or nuclear staining with propidium iodide (PI) to detect DNA fragmentation and nuclear condensation, respectively. Images were taken by confocal laser scanning microscopy.*

TF2 and **TF3** induced small populations of annexin V-positive cells (that express a higher concentration of phosphatidylserine in the outer cell membrane, an early marker of apoptosis) in both HSC-2 and HSC-4 cells (Figure 4A). HSC-2 cells were positive both for annexin V and PI staining, suggesting the occurrence of necrosis in this cell line. On the other hand, HSC-4 cells were positive only for annexin-V, but not with PI, suggesting the occurrence of early apoptosis in this cell line.

TF2 and **TF3** induced the formation of acidic organelles (stained by acridine orange) (Figure 4B) and the accumulation of LC3 in the autophagosome in the cytoplasm of both HSC-2 and HSC-4 cells (Figure 4C), and such changes were canceled by the addition of 3-methyladenine (3-MA), an inhibitor of autophagy (Figure 4B, 4C). 3-MA also reduced the cytotoxicity of **TF2** and **TF3** (Figure 5).

Discussion

We demonstrate that α-trifluoromethyl acyloins (**TF2** and **TF3**) did not induce apoptosis (type I programmed cell death) in HSC-4 cells, based on their inability to induce apoptosis markers such as DNA fragmentation (assessed by TUNEL method) and caspase-3 activation (assessed by substrate cleavage assay). This finding is quite different from the previous report that **TF2** and **TF3** induced DNA fragmentation (as identified by TUNEL method) and activation of caspases (as identified by degradation products of cytokeratin 18 with M30 monoclonal antibody) in HSG cells (8). Furthermore, the detection of degradation product with M30 monoclonal antibody is an indirect method for the evaluation of caspase activation. Therefore, further criteria of apoptosis should be assayed in HSG cells.

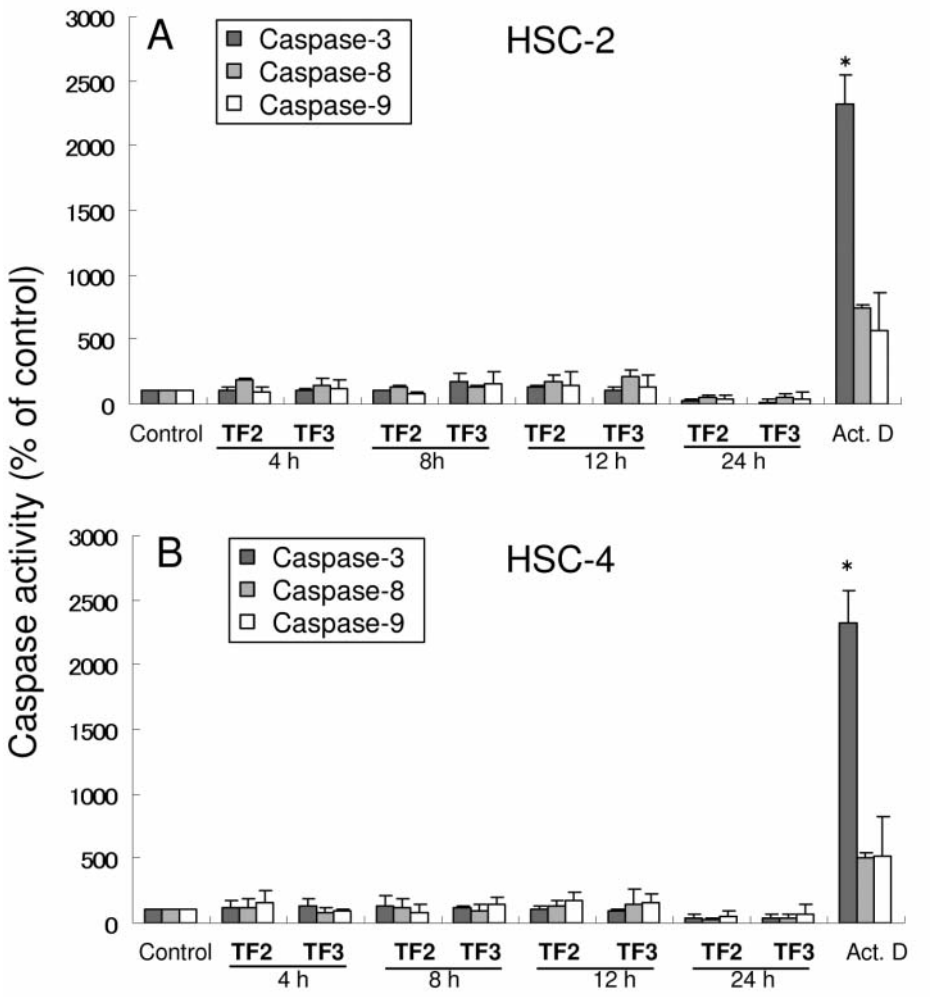

Figure 3. *Failure of **TF** compounds to activate caspase-3, -8 and -9. HSC-2 and HSC-4 cells were incubated for 4, 8, 12 and 24 hours without (control) or with the indicated concentrations of **TF2**, **TF3**, or actinomycin D (1 μg/ml) (Act. D, a positive control), and the activities of caspase-3, -8 and -9 were assayed by substrate cleavage assay. Caspase activity was expressed as % of the untreated control. *p<0.05.*

TF2 or **TF3** treatment produced annexin V-positive/PI-positive HSC-2 cells and annexin V-positive/PI-negative HSC-4 cells at early stages, suggesting the occurrence of transient necrosis or apoptosis in these cell lines, respectively. However, **TF2** and **TF3** induced little or no DNA fragmentation and failed to induce the activation of caspase-3, caspase-8 and caspase-9 in both HSC-2 and HSC-4 cells at later stages, suggesting the incidence of non-apoptotic cell death at later stages. Autophagy (type II programmed cell death) (15, 16) is a pathway for the bulk degradation of subcelluar constituents through the creation of autophagosomes/autolysosomes in response to stresses such as nutrient deprivation (17-19). In general, autophagy is utilized so that cells can survive, but constitutive activation of autophagy may induce cell death. We therefore investigated the possibility that **TF2** and **TF3** induce autophagy using 3-MA, an inhibitor of autophagy (16). We found that this inhibitor was cytotoxic to both HSC-2 and HSC-4 cells, and when corrected for its cytotoxicity, 3-MA considerably reduced the cell death induced by **TF2** and **TF3**. We also found that **TF2** and **TF3** induced the accumulation of autophagy-specific markers such as LC3 in the autophagosome (20) and the formation of the acidic organelles (stained by acridine orange) in both of these cell lines. All these data support the possibility of the induction of autophagy.

It has been reported that brain tumor cell lines are easily susceptible to autophagy upon treatment with various chemotherapeutic agents (21). This suggests that the type of cell may be one of the factors that determines the type of cell death (22-24). Another factor that may determine the type of cell death is the chemical structure of the test compound (25).

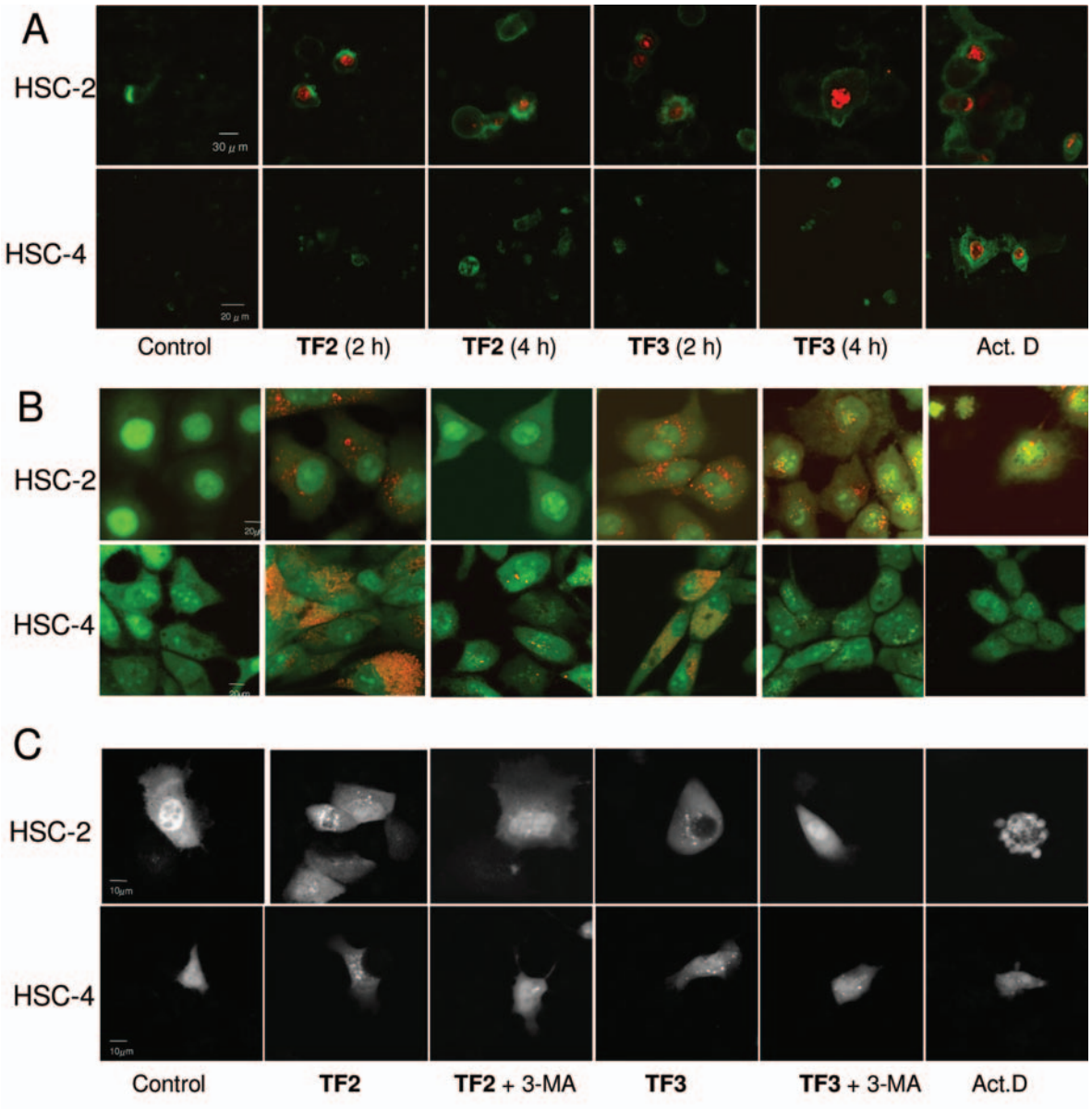

Figure 4. *Detection of early apoptosis marker (A), the formation of acidic organelles (B) and autophagosome (C) after treatment with* ***TF2*** *or* ***TF3*** *in HSC-2 and HSC-4 cells. A, Cells were treated for 2 or 4 hours with 200 μM* ***TF2*** *or* ***TF3****, or for 2 hours with 1 μg/ml actinomycin D (Act. D), and then stained with annexin V and PI. B, Cells were pretreated with or without 3-methyladenine (3-MA) (10 mM) for 30 min, followed by treatment for 4 hours with 200 μM* ***TF2****, 200 μM* ***TF3,*** *or 1 μg/ml Act. D. The cells were then stained with acridine orange and the formation of acidic organelles was observed under confocal laser scanning microscopy. C, Cells were transiently transfected with LC3-GFP expression vector. Cells were pretreated with or without 3-methyladenine (3-MA) (10 mM) for 30 min, and then treated for 4 hours with 200 μM* ***TF2****, 200 μM* ***TF3,*** *or 1 μg/ml Act. D. The accumulation of LC3-GFP in the autophagosome was observed under confocal laser scanning microscopy.*

For example, we reported that α,β-unsaturated ketones, such as 2-cyclohexen-1-one, 2-cyclopenten-1-one, 4,4-dimethyl-2-cyclopenten-1-one, 2-cyclohepten-1-one, α-methylene-γ-butyrolactone, 5,6-dihydro-2*H*-pyran-2-one and methyl 2-oxo-2*H*-pyran-3-carboxylate (12) activated caspases-3, -8 and -9 very weakly. Our data strongly suggest that α-hydroxyl ketones such as **TF2** and **TF3** induce autophagy following transient necrosis or apoptosis at an early stage in HSC-2 and HSC-4 cells, respectively. Further studies are required to elucidate the mechanism of heterogenic response of human oral squamous cell carcinoma cell lines to autophagy inducers.

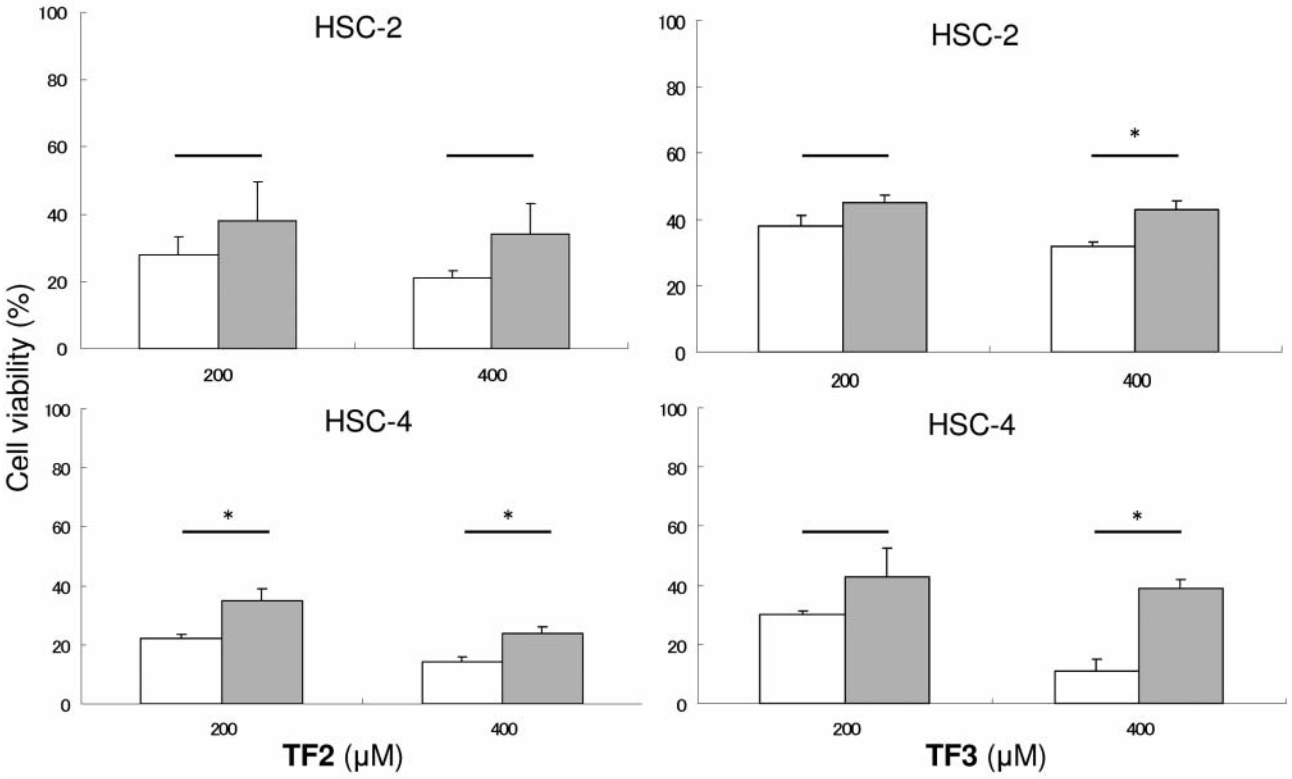

Figure 5. *Effect of 3-methyladenine on **TF**-induced cytotoxicity. HSC-2 and HSC-4 cells were pretreated with (gray bar) or without (white bar) 3-MA (10 mM) for 30 min, and then incubated for a further 24 hours without (control) or with the indicated concentration of **TF2** or **TF3**, and the viable cell number was determined by MTT assay. Data were expressed as % of that of the control culture. Each value represents the mean from three independent experiments. *p<0.05.*

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