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Slow Rise of Ca^{2+} and Slow Release of Reactive Oxygen Species are Two Cross-Talked Events Important in Tumour Necrosis Factor-α-Mediated Apoptosis

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Tumour necrosis factor-α (TNF-α) was found to be a cell cycle-independent apoptogenic cytokine in cultured fibroblast L929 cells. This assertion is based on the observations (1) TNF-α increased the number of cells with hypo-diploid DNA in a time dependent manner as revealed by flow cytometry, and (2) TNF-α induced DNA fragmentation as resolved by agarose gel electrophoresis. When cells were exposed to TNF-α (50 ng/ml), a slow rise in intracellular free Ca^{2+} level and a delayed increase in the production of reactive oxygen species (ROS) (both observed 3 h after the addition of TNF-α) were observed in fluo-3 and fura-red or dichlorofluorescein loaded cells, respectively. Interestingly, challenge of cells with TNF-α in the presence of BAPTA/AM, an intracellular Ca^{2+} chelator, decreased the release of ROS. Removal of ROS by 4-hydroxy 2,2,6,6-tetra-methyl-piperidinooxy (4OH-TEMPO) blocked the TNF-α-mediated Ca^{2+} rise. Moreover, when cells were exposed to TNF-α with both 4OH-TEMPO and BAPTA/AM, more viable cells were found than from treatment with either BAPTA/AM or 4OH-TEMPO. These results suggest that ROS and cellular Ca^{2+} are two cross-talk messengers important in TNF-α-mediated apoptosis.

Keywords: TNF-α, L929 cells, apoptosis, cross talk, ROS, Ca^{2+}

Abbreviations: TNF-α, murine recombinant tumour necrosis factor-α; $[Ca^{2+}]i$, intracellular free calcium concentration; DCF, dichlorofluorescein; 4OH-TEMPO, 4-hydroxy 2,2,6,6-tetra-methyl-piperidinooxy; BAPTA/AM, [1,2-bis(2)amino-phenoxy]ethane-N,N,N^1,N^1-tetra-acetic, acetymethyl ester; ROS, reactive oxygen species; Nac, N-acetyl-L-cysteine

INTRODUCTION

Tumour necrosis factor-α (TNF-α) is a pleiotropic cytokine primarily produced by activated macrophages. Different mechanisms have been suggested to contribute to the TNF-α-mediated cell killing.[1,2] These include the generation of reactive oxygen species (ROS),[3,4] activation of phospholipase and release of arachidonic acid,[5,6] activation of protease and caspases[7,8] and

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impairment of mitochondrial functions.[9–11] However, the mechanism of TNF-α cytotoxicity is not fully understood. Even the mode of cell death is still controversial. Necrosis and apoptosis were reported in murine L929 cells after TNF-α treatment.[12,13]

Considering the important role of cellular Ca^{2+} in apoptosis and necrosis mediated by other death-inducing agents,[14] the role of Ca^{2+} in the TNF-α-mediated cytotoxicity is unclear. Results in U937 lymphoma indicate that neither an immediate increase in cellular Ca^{2+} level nor Ca^{2+} influx was found after TNF-α treatment.[15] However, treating L929 cells with TNF-α with LiCl induces an increase in the accumulation of inositol trisphosphate, and cytotoxicity suggests a role of intracellular Ca^{2+} in the TNF-α-mediated cell death.[16] Evidence also indicates a positive role of intranuclear [Ca^{2+}] in TNF-α-mediated cell death in mammary adenocarcinoma cells.[17] Here, we investigate the role of Ca^{2+} in TNF-α-mediated cytotoxicity in L929 cells, with emphasis on the effect of Ca^{2+} on ROS production.

MATERIALS AND METHODS

Materials and Cell Culture

Recombinant murine TNF-α was purchased from Boehringer Mannheim (Mannheim, Germany). The diacetate form of dichlorofluorescein (DCF-DA), fluo3/AM, fura-red/AM and BAPTA/AM were from Molecular Probes (Eugene, USA). Other reagents were from Sigma (St. Louis, USA). L929 cells, a TNF-α sensitive cell line from the American Type Culture Collection, were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (Gibco, Gaithersburg, USA) at 37°C, 5% CO_2. The generation time of L929 cells is about 36 h at 37°C, 5% CO_2. Cells after treatments were trypsinized and all the cells (adherent and non-adherent) were collected for flow analysis.

Isolation and Electrophoresis of DNA

L929 cells (1×10^6/ml) treated with TNF-α or medium were pelleted by centrifugation and resuspended in 270 µl pre-cooled cell lysis buffer (in mM: 10 Tris/HCl, 10 NaCl, 10 EDTA, pH 7.4) with 30 µl 10% SDS. Subsequently, Proteinase K was added to a final concentration of 100 µg/ml. After incubation overnight at 37°C for complete digestion, DNA was extracted twice with equal volumes of phenol followed by two extractions with chloroform. The DNA was then further extracted by two volumes of absolute ethanol followed by centrifugation at 13,000 rpm for 10 min.

Extracted DNA was resuspended in 40 µl TE buffer (in mM: 10 Tris/HCl, 1 EDTA, pH 7.6). Loading buffer (10 mM EDTA, 0.25% bromophenol blue, 30% glycerol) was then added at a 1 : 5 ratio. Samples were then loaded onto a 1.5% agarose gel and electrophoresis was carried out at 50 V in TBE buffer (in mM: 2 EDTA, 90 Tris/HCl, 90 boric acid, pH 8.0). One hundred base-pair ladder molecular weight markers (Gibco) were used. After electrophoresis, DNA was visualized by soaking the gel in TBE buffer containing 1.5 µg/ml ethidium bromide.

Cell Cycle Analysis

L929 cells incubated with TNF-α or medium alone were washed and fixed with 70% ethanol overnight. After centrifugation, cells were rehydrated in PBS for 30 min in the presence of RNase A (8 µg/ml) for 20 min. Subsequently, cells were labelled with propidium iodide (PI) (40 µg/ml) and analysis was carried out with a flow cytometer (FACSort, Becton Dickinson). Laser excitation was set at 488 nm and signals for PI were obtained with a 570 nm band pass filter.

ROS Determination by Flow Cytometry

The release of ROS was measured by flow cytometry with DCF-DA. Briefly, cells treated with different agents were suspended in PBS (5×10^5/ml) and incubated with DCF-DA (final

concentration 10 μM) at room temperature. Cells were then submitted to flow analysis with FACSort (Becton Dickinson). Forward and side scatters were used to establish size gates and exclude debris from the analysis. The excitation wavelength was 488 nm while the emitted fluorescence was collected at 510 nm. For one single analysis, the fluorescence properties of 10,000 cells within the gates were collected.

Ca^{2+} Determination by Flow Cytometry

The intracellular free Ca^{2+} level ($[Ca^{2+}]i$) was determined by flow cytometry with fluo-3 and fura-red to increase the resolution of the change of ($[Ca^{2+}]i$). Briefly, cells treated with different agents were suspended in PBS (5×10^5/ml) and incubated with fura-red/AM (final concentration 10 μM) and fluo-3/AM (final concentration 10 μM) at room temperature for 1 h. After washing, cells were submitted to flow analysis with FACSort (Becton Dickinson). Forward and side scatters were used to establish size gates and exclude debris from the analysis. The excitation wavelength was 488 nm while the emitted fluorescence was determined at 510 nm for fluo-3 and 570 nm for fura-red.

Neutral Red Assay

L929 cells (3×10^4/well) in a 96-well plate were treated with different agents as indicated at 37°C, 5% CO_2. After incubation, cells were washed twice and neutral red (0.5% w/v, 50 μl/well) was added. After 1 h incubation, cells were washed and the wells were air-dried. Subsequently, cells were lysed with 1% SDS and absorbance was determined at 540 nm.

RESULTS

Murine Recombinant TNF-α Induced Apoptosis in L929 Cells

L929 cells have long been used as a model cell line to examine the cytotoxic action of recombinant human TNF-α. Figure 1 shows the effect of murine recombinant TNF-α on the induction of apoptosis in L929 cells. It is clear in Figure 1(a)–(d) that incubation of cells with TNF-α (50 ng/ml) from 15 min to 6 h did not alter much the pattern of the cell cycle. When cells were incubated with TNF-α for 10 h, a cell population with a DNA content below a normal G0/G1 value was found (Figure 1(e)). Cells at all phases in the cell cycle were affected. Under identical conditions, a time-dependent DNA fragmentation was observed by agarose gel electrophoresis (Figure 1(f)). These observations unequivocally indicate that murine recombinant TNF-α is not an immediate death

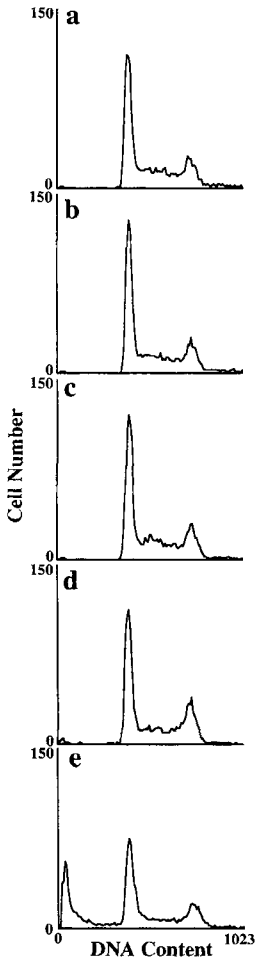

FIGURE 1(a)–(e)

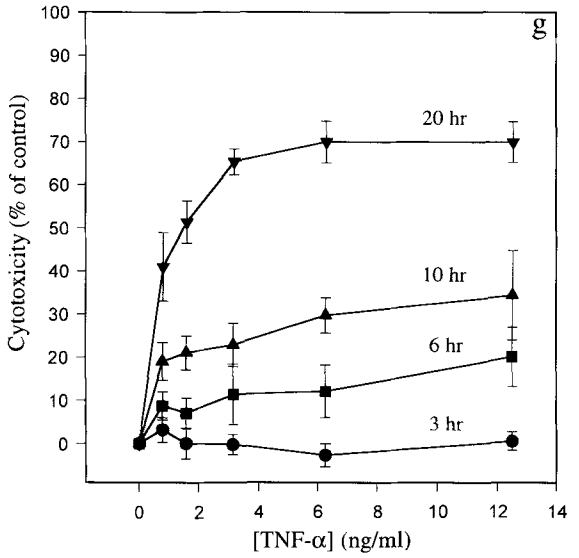

FIGURE 1(f) and (g)

FIGURE 1 Effect of TNF-α on the induction of apoptosis in L929 cells. L929 cells (1×10^6/ml) were treated with medium alone (a) or TNF-α (50 ng/ml) at 37°C, 5% CO_2 for 15 min (b), 3 h (c), 6 h (d) or 10 h (e). After treatment, cells were fixed and stained with propidium iodide (43 µg/ml) for 30 min. The DNA content was then determined by flow cytometry. (f) DNA from cells (1×10^6/ml) treated with medium or TNF-α (50 ng/ml) as indicated was extracted and analysed by agarose gel electrophoresis. (g) L929 cells (3×10^5/ml) were treated with various concentrations of TNF-α at 37°C, 5% CO_2 for 3 h (●), 6 h (■), 10 h (▲) or 20 h (▼). Subsequently, cytotoxicity was determined by neutral red uptake assay. Results are mean ± SD for 7 determinations. Data shown here are from a single experiment representative of several independent experiments.

inducer in L929 cells, but requires time to initiate a death programme. As shown in Figure 1(g), the longer was the incubation time with TNF-α, the higher the cytotoxicity was observed. Due to its simplicity, this neutral red uptake assay was used in the later experiments.

TNF-α Increased the Cellular ROS Content

Growing evidence indicates that ROS play a key role in apoptosis.[18,19] To determine the release of ROS in TNF-α-treated cells, the DCF-DA was used to monitor the formation of intracellular ROS.[19] The oxidation of the non-fluorescent DCF-DA by ROS yields the fluorescent form of DCF and the fluorescence of the intracellularly accumulated DCF therefore represents the amount of ROS released. With an aid of a flow cytometer, the amount of ROS released from individual cells was studied. As shown in Figure 2(a), incubation of cells with TNF-α (50 ng/ml) for 15 min did not produce significant change in the ROS content as compared to control. However, when cells were treated with TNF-α (50 ng/ml) for a longer time, the DCF pattern shifted to the right hand side as compared to their corresponding control (Figure 2(b)–(d)). The longer was the incubation time with TNF-α, the more ROS was released. In the 10 h treatment group (Figure 2(d)), a population of higher responder on the right and a lower responder on the left in terms of DCF fluorescence were found. The occurrence of the two populations was not clear in the 6 h treatment (Figure 2(c)). When the lower responders in the 10 h treatment were sorted out for the determination of viability, all of them were dead cells

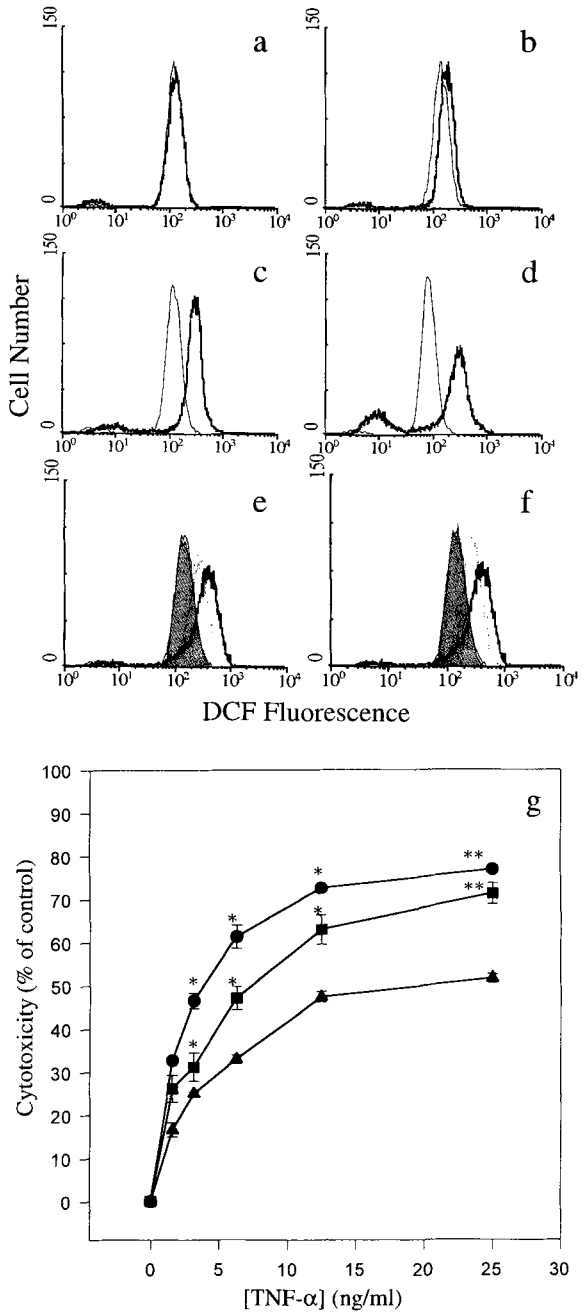

FIGURE 2 Generation of ROS by TNF-α in L929 cells. L929 cells (1×10^6/ml) were treated with medium alone (hair line), or TNF-α (50 ng/ml) (thick line) at 37°C, 5% CO_2 for 15 min (a), 3 h (b), 6 h (c) or 10 h (d). L929 cells were also treated with medium alone (hair line), Nac (20 mM) (curve with grey area), TNF-α (50 ng/ml) (thick line) or TNF-α (50 ng/ml) together with Nac (20 mM) (dotted line) at 37°C, 5% CO_2 for 6 h (e). Treatments in (f) were similar to (e) except that Nac was replaced with 4OH-TEMPO (5 mM). The release of ROS was then determined by flow cytometry with DCF. Data shown here are from a single experiment representative of several experiments. (g) L929 cells (3×10^5/ml) were treated with various concentrations of TNF-α in the absence (●) or presence of Nac (20 mM) (■) or 4OH-TEMPO (5 mM) (▲) at 37°C, 5% CO_2 for 20 h. Cytotoxicity was then determined by neutral red uptake assay. Results are mean ± SD for 7 determinations. The cytotoxicity of cells treated with Nac or 4OH-TEMPO was ~80% of the one treated with medium alone. $^*P < 0.01$, $^{**}P < 0.05$.

(data not shown). Results in Figure 2(a)–(d) therefore suggest that the release of ROS might be a mediator for the induction of apoptosis.

Next, the effect of antioxidants such as N-acetyl-L-cysteine (Nac) and 4-hydroxy 2,2,6,6-tetramethylpiperidinooxy (4OH-TEMPO) on the TNF-α-mediated ROS production was examined. Nac and 4OH-TEMPO are known as ROS scavengers.[20] Treating cells with Nac (20 mM) (Figure 2(e)) or 4-OH-TEMPO (5 mM) (Figure 2(f)) alone did not alter the release of ROS when compared to the untreated group. In the presence of these antioxidants, the level of ROS production elicited by TNF-α (50 ng/ml, 6 h) was greatly reduced (Figure 2(e) and (f)). This suppressive effect was not due to a 'too-early' toxicity since protections by Nac and 4OH-TEMPO was observed in the viability assay (Figure 2(g)). These results thus suggest that ROS was important in the TNF-α-mediated cytotoxicity. However, although the protective effect of 4OH-TEMPO seemed to be greater than that of Nac (Figure 2(g)), it could not totally remove the TNF-α cytotoxicity. This suggests that mechanisms other than the formation of ROS might be involved in the TNF-α apoptosis.

TNF-α Induced the Rise of Cellular $[Ca^{2+}]$

The effect of TNF-α on the intracellular free Ca^{2+} level ($[Ca^{2+}]i$) was also investigated. In this study, two fluorescent Ca^{2+} indicators, fluo-3 and fura-red, were loaded into L929 cells to monitor the change of $[Ca^{2+}]i$. Simultaneous loading of cells with fura-red and fluo-3 which exhibit reciprocal shifts in fluorescence intensity upon

binding with Ca^{2+} provides a better resolution of the change of $[Ca^{2+}]i$ after stimulation.[21] As shown in Figure 3(a), each dot in the dot-plot represents the fluorescence intensities of fluo-3 and fura-red of a single cell. In the resting stage, most of the dots were found in the lower part of the dot-plot (Figure 3(a)). When the $[Ca^{2+}]i$ increases, more dots will theoretically move anti-clockwisely to the upper left quadrant. This phenomenon was observed when L929 cells were incubated with TNF-α (50 ng/ml) for 3 h (Figure 3(a) and (b)), 6 h (Figure 3(c) and (d)) or 10 h (Figure 3(e) and (f)). Similar to the ROS responses, the longer was the incubation time with TNF-α, the higher was the $[Ca^{2+}]i$ observed. Again, the role of cellular Ca^{2+} in the cytotoxicity was investigated with the cellular Ca^{2+} modulators. As depicted in Figure 3(g) and (h), pre-treatment of cells with thapsigargin (2 μM), an inhibitor that blocks the microsomal Ca^{2+}-ATPase activity,[22] for 10 h altered $[Ca^{2+}]i$ a little. When cells were treated simultaneously with both thapsigargin (2 μM) and TNF-α (50 ng/ml), the $[Ca^{2+}]i$ was much higher than when treated with TNF-α alone (Figure 3(f) and (h)). In the presence of thapsigargin, the cytotoxic effect elicited by TNF-α was more pronounced too (Figure 3(i)). These results suggested that a marked increase in $[Ca^{2+}]i$ promotes the TNF-α cytotoxic effect.

Relationship Between the Rise of $[Ca^{2+}]i$ and the Release of ROS in TNF-α-Treated L929 Cells

The rise of $[Ca^{2+}]i$ and the release of ROS by TNF-α were both a slow process. However, the relationship between these two cellular messengers under TNF-α challenge was not clearly defined. We therefore examined the relationship between ROS and $[Ca^{2+}]i$. As shown in Figure 4(a) and (b), incubation of cells with 4OH-TEMPO (5 mM) for 10 h only produced a little change in the $[Ca^{2+}]i$. Interestingly, incubation of cells with TNF-α (50 ng/ml) in the presence of 4OH-TEMPO (5 mM) for 10 h reduced the TNF-α-mediated $[Ca^{2+}]i$ rise (Figure 4(c)–(d)). This suppressive effect was not due to an early death since the introduction of ionomycin (8 μg/ml) to the cells treated with TNF-α and 4OH-TEMPO at the end of the experiment could produce an abrupt rise in $[Ca^{2+}]i$ (Figure 4(e)–(h)). The spur and the slope of dots in Figure 4(g) also indicate that pre-treating cells with TNF-α potentiated the ionomycin-induced Ca^{2+} rise.

With the use of BAPTA/AM, an intracellular Ca^{2+}chelator[23], the role of $[Ca^{2+}]i$ in inducing the release of ROS was also investigated.

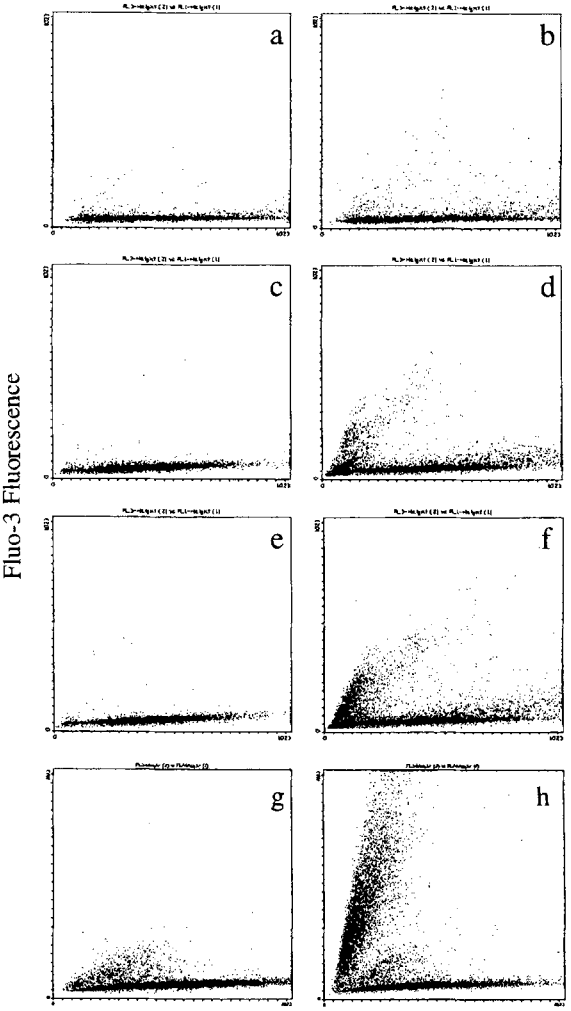

FIGURE 3(a)–(h)

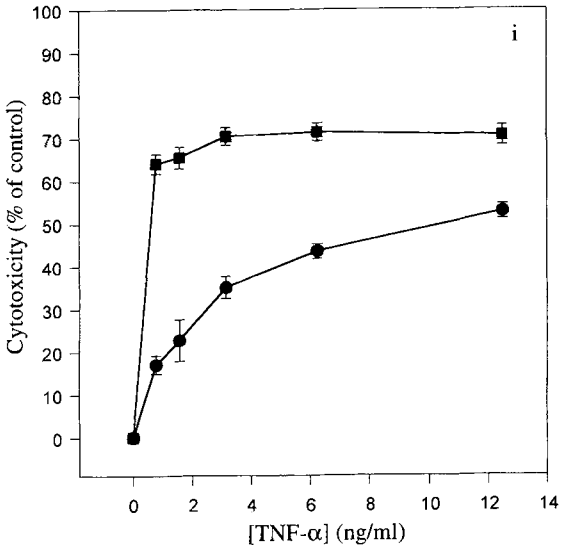

FIGURE 3(i)

FIGURE 3 Effect of TNF-α on the rise of $[Ca^{2+}]i$ in L929 cells. L929 cells (1×10^6/ml) were treated with medium alone (left panels) or TNF-α (50 ng/ml) (right panels) at 37°C, 5% CO_2 for 3 h (a, b), 6 h (c, d) or 10 h (e, f). L929 cells were also treated with thapsigargin (2 μM) (g) or thapsigargin (2 μM) together with TNF-α (50 ng/ml) (h) at 37°C, 5% CO_2 for 10 h. Cells were then loaded with fluo-3/AM (10 μM) and fura-red/AM (10 μM) for 1 h and the fluorescence of fluo-3 and fura-red were determined by a flow cytometer. Each dot in the dot-plot represents the fluorescence intensities of the two indicators in one single cell. Data shown here are from a single experiment representative of several experiments. (i) L929 cells (3×10^5/ml) were treated with various concentrations of TNF-α in the absence (●) or presence of thapsigargin (2 μM) (■) at 37°C, 5% CO_2 for 20 h. Cytotoxicity was then determined by neutral red uptake assay. Results are mean ± SD for 7 determinations. The cytotoxicity of cells treated with thapsigargin was ~70% of the one treated with medium alone.

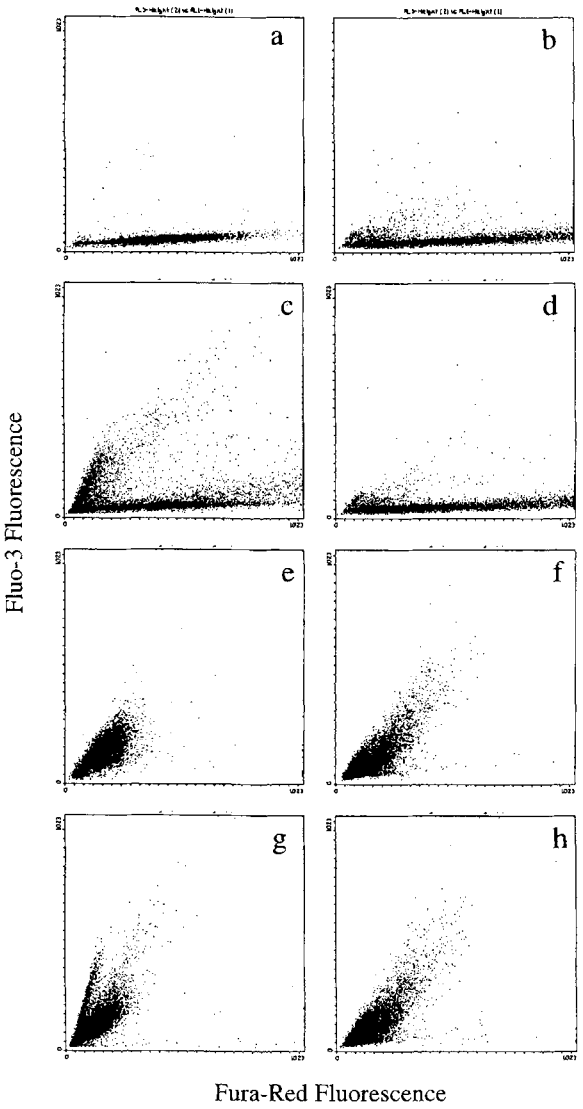

FIGURE 4 Effect of 4OH-TEMPO on the TNF-α-mediated increase in $[Ca^{2+}]i$. L929 cells (1×10^6/ml) were treated with medium alone (a, e), 4OH-TEMPO (5 mM) (b, f), TNF-α (50 ng/ml) (c, g), or 4OH-TEMPO (5 mM) together with TNF-α (50 ng/ml) (d, h) at 37°C, 5% CO_2 for 10 h. Cells were then loaded with fluo-3/AM (10 μM) and fura-red/AM (10 μM) for 1 h. After washing, the fluorescence of fluo-3 and fura-red were determined by a flow cytometer. Cells under different treatments were subsequently stimulated with ionomycin (5 μM) for 10 min at room temperature and the fluorescence was then determined (e–h).

Interestingly, loading cells with BAPTA/AM suppressed the TNF-α-elicited release of ROS while BAPTA itself did not alter much the release of ROS at the resting state (Figure 5). With a longer incubation time with TNF-α and BAPTA/AM, a stronger suppression was observed (Figure 5). Again, the effect of BAPTA and 4OH-TEMPO on the TNF-α-mediated cytotoxicity was examined. As shown in Figure 6, treating cells with either BAPTA/AM (10 μM) or 4OH-TEMPO (5 mM) could block the TNF-α-mediated cytotoxicity. The protection elicited by BAPTA/AM was weaker than that elicited by 4OH-TEMPO. However, incubation of cells with both BAPTA/AM and 4OH-TEMPO produced a stronger protective effect in rescuing more cells from the TNF-α mediated cytotoxicity (Figure 6). These results

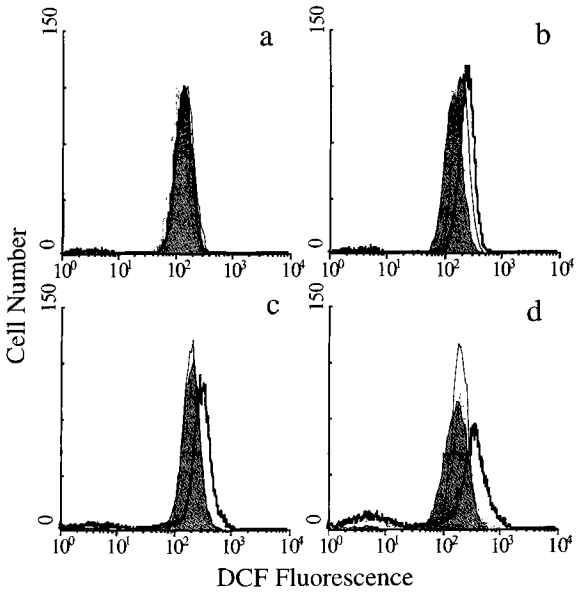

FIGURE 5 Effect of BAPTA/AM on the TNF-α-elicited generation of ROS in L929 cells. L929 cells (1×10^6/ml) were treated with medium alone (hair line curve), BAPTA/AM (10 μM) (curve with grey area), TNF-α (50 ng/ml) (thick line) or TNF-α (50 ng/ml) together with BAPTA/AM (10 μM) (dotted line) at 37°C, 5% CO_2 for 15 min (a), 3 h (b), 6 h (c) and 10 h (d). The release of ROS was then determined by flow cytometry with DCF. Data shown here are from a single experiment representative of several independent experiments.

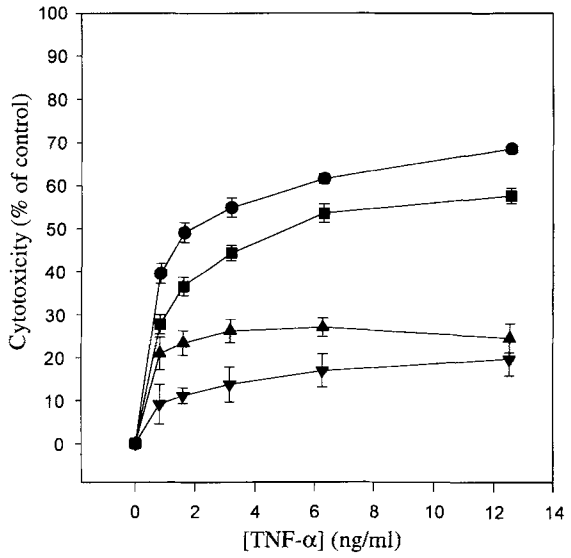

FIGURE 6 Effect of Ca^{2+} chelator and anti-oxidant on the TNF-α-elicited cytotoxicity in L929 cells. L929 cells (3×10^5/ml) were treated with various concentrations of TNF-α in the absence (●) or presence of BAPTA/AM (10 μM, 2 h) (■), 4OH-TEMPO (5 mM) (▲) or 4OH-TEMPO (5 mM) together with BAPTA/AM (10 μM, 2 h) (▼) at 37°C, 5% CO_2 for 20 h. Cytotoxicity was then determined by neutral red uptake assay. Results are mean ± SD for 7 determinations. The cytotoxicity of cells treated with 4OH-TEMPO and BAPTA was ~75% of the one treated with medium alone. Data shown here are from a single experiment representative of several independent experiments.

thus indicate that both the Ca^{2+} and ROS are executors for the TNF-α cytotoxic actions. Moreover, upon TNF-α stimulation, the slow rise of $[Ca^{2+}]i$ was a promoting factor on the release of ROS and vice versa.

DISCUSSION

TNF-α alone exerts cytotoxic effect against a number of tumour cells including L929 cells. As mentioned earlier, TNF-α was found to induce necrosis[1] or apoptosis.[2] In our study, the occurrence of a hypo-dipoid peak and DNA fragmentation (Figure 1) support the notion that TNF-α induces apoptosis in L929 cells. Moreover, that translocation of phosphatidyl-serine to the outer plasma membrane without the loss of membrane integrity was observed in the TNF-α treated L929 cells further supports our conclusion (data not shown).

To initiate and trigger apoptosis, cellular Ca^{2+} was demonstrated to be a key signal molecule in many experimental studies.[14] However, the role of Ca^{2+} in the TNF-α-mediated cytotoxicity is controversial (see Introduction). In the present study, we showed a slow increase in $[Ca^{2+}]i$ in L929 cells after TNF-α treatment (Figure 3). This finding is consistent with our previous observation that no rapid change in $[Ca^{2+}]i$ was found immediately after the addition of TNF-α.[24] With the use of thapsigargin to inhibit the Ca^{2+} pump to maintain a high $[Ca^{2+}]i$ (Figure 3(g)–(h)), a more pronounced cytotoxicity was observed after TNF-α treatment (Figure 3(i)). In agreement with this finding, chelating cellular Ca^{2+} by BAPTA/AM could alleviate the TNF-α-elicited cytotoxicity (Figure 6). These results thus provide

strong evidence for the important role of cellular Ca^{2+} in the TNF-α-mediated cytotoxicity.

The role of ROS in the TNF-α-mediated cell death has been postulated by many laboratories.[25,26] Our study confirms the fact that TNF-α produced ROS (Figure 2). The enhancement of the release of ROS by TNF-α was not a rapid process. It took 6 h for a significant increase. Removing ROS by Nac or 4OH-TEMPO reduced both the release of ROS (Figure 2(e) and (f)) and cytotoxicity (Figure 2(g)) after TNF-α treatment. These observations suggest that the release of ROS is also an important mediator in L929 cells leading to cell death after incubation with TNF-α.

Interestingly, chelating the cellular Ca^{2+} by BAPTA/AM was able to reduce the release of ROS (Figure 5). Similarly, removing ROS by 4OH-TEMPO reduced the rise of $[Ca^{2+}]$ (Figure 4). These suppressive effects could be explained by an early cell death and therefore no apparent increase in $[Ca^{2+}]i$ nor release of ROS was observed when cells were treated with TNF-α in the presence of 4OH-TEMPO or BAPTA/AM. However, that exposure of cells to TNF-α together with either 4OH-TEMPO or BAPTA/AM gave a higher viability (Figure 6) eliminates this possibility. In fact, more viable cells were found when both 4OH-TEMPO and BAPTA/AM were used. For our curiosity, we had challenged the cells with ionomycin and freshly prepared H_2O_2, however, no change in the release of H_2O_2 and $[Ca^{2+}]i$ was observed respectively (data not shown). These results imply that the death programme induced by TNF-α is a complicated pathway. Increase in the $[Ca^{2+}]i$ alone is not sufficient to stimulate the rise of H_2O_2 and vice versa.

In conclusion, our results indicate that TNF-α is an apoptotic agent in L929 cells, which did not cause an immediate but a slow increase in cellular $[Ca^{2+}]$ and ROS. Interestingly, application of Ca^{2+} chelator BAPTA/AM suppressed the formation of ROS and the use of antioxidant, 4OH-TEMPO, blocked the rise of $[Ca^{2+}]i$. These results indicate a cross-talk and a possible amplifying loop between cellular Ca^{2+} and ROS in L929 cells after TNF-α stimulation.

Acknowledgements

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