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TUMOR NECROSIS FACTOR-α SIGNALING CASCADES IN APOPTOSIS, NECROSIS, NECROPTOSIS AND CELL PROLIFERATION

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ABSTRACT

Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine involved in host defense, inflammation, apoptosis, autoimmunity, organogenesis and lymphoid microarchitecture. Many of these activities may be explained by the ability of this cytokine to induce distinct signal transduction pathways that recruit regulatory proteins involved in differentiation, cell death or cell proliferation. In this review, we discuss the contribution of caspases -3, -6, -7 and -8, and of cyclin-dependent kinases (CDKs), cyclin B and cyclin-dependent kinase inhibitors (CKI p21 and p27), as well as retinoblastoma tumor suppressor in the signaling cascades triggered by TNF-α to induce apoptosis, necrosis and cellular proliferation in the murine cell lines NIH3T3 and WEHI-164 and the human cervical carcinoma cell line HeLa-S3. Based on the findings of many literature reports and our own data, we discussed a model in which caspases are continuously activated throughout the cell cycle and kept at a critical threshold level by IAP (inhibitor of apoptosis) antagonists. Following the release of Smac/Diablo and HtrA2/OMI from mitochondria in response to diverse stimuli, this threshold is overcome and results in amplified caspase activation and cell death. An alternative, caspase-independent mechanism of cell death is induced in NIH3T3 fibroblasts by a combination of TNF and the pan-caspase inhibitor z-VAD-fmk. This cell death phenotype, known as necroptosis, displays some morphological features of apoptosis and necrosis. Although caspases are critical regulators of the TNF signaling pathway during cellular life and death, the mechanisms involved in the fine regulation of their dual effects remain to be fully elucidated.

Key words: Apoptosis, caspases, cell cycle, necrosis, oncogenes, tumor necrosis factor

INTRODUCTION

Tumor necrosis factor-α (TNF-α) is a multifunctional cytokine involved in a variety of cellular responses, including the induction of apoptosis and necrosis in many cancer cell lines and cellular proliferation in normal B and T cells and fibroblasts [17]. TNF-α binds to two distinct cell surface receptors of 55 kDa (TNFR1) and 75 kDa (TNFR2). TNFR1 contains a death domain (DD) that is shared with other death receptors (DRs) such as CD95 (Fas, APO-1), TNF-related apoptosis-inducing receptor (TRAIL), DR4 and DR5. The binding of TNF-α to TNFR1 results in receptor trimerization, clustering and internalization of activated TNF/TNFR1 complexes (TNF receptosomes). This event allows the binding of an intracellular adapter molecule called TRADD (TNFR-associated death domain) via interaction between death domains, and is followed by the recruitment of the adapter protein Fas-associated death domain protein (FADD) and, subsequently, pro-caspases -8 and -10 to establish the death-inducing signaling complex (DISC). The resulting oligomerization of pro-caspase-8 results in its autoprocessing and activation [42,50,53]. Caspase-8

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ABBREVIATIONS

AIF, apoptosis-inducing factor; Apaf-1, apoptotic protease activating factor; BH, BCL-2 homology; BIR, baculoviral IAP repeat; BCL-2, B cell lymphoma; CARD, caspase recruitment domain; CDK, cyclin dependent kinase; CKI, cyclin-dependent kinase inhibitors; DD, death domain; DED, death effector domains; DISC, death-inducing signaling complex; DR, death receptor; EGF, epidermal growth factor; Endo G, endonuclease G; FADD, Fas-associated protein with a death domain; IAP, inhibitor of apoptosis protein; JNK, c-jun N-terminal kinase or stress-activated protein kinase; NF-κB, nuclear factor-κB; PDGF, platelet-derived growth factor; RAIDD, RIP-associated ICH-1; RIP, adapter receptor interacting protein; ROS, reactive oxygen species; TNF, tumor necrosis factor; TRADD, TNF receptor associated protein, TRAF, TNF receptor-associated factor, TRAIL, TNF-related apoptosis-inducing ligand.

then cleaves and activates downstream caspases, such as caspases -3, -6 and -7, which initiate the proteolytic cleavage of numerous nuclear, cytoplasmic and structural proteins to induce apoptosis [14,42]. TNFR1 can also mediate apoptosis through the recruitment of an adapter molecule called RAIDD (RIP-associated ICH-1). RAIDD associates with RIP through death domain interactions and then recruits caspase 2 through interaction with a motif similar to the death effector domain known as CARD (caspase recruitment domain). The recruitment of caspase 2 leads to the induction of apoptosis [14,17,50,53].

The signaling initiated by TNFR1 is also linked to protection against apoptosis. TNFR1 activation of JNK (c-jun N-terminal kinase or stress-activated protein kinase) requires the signal adapter TNF-receptor associated factors TRAF1 and TRAF2 and the adapter receptor interacting protein (RIP). TRAF 2 and RIP initiate a separate downstream pathway that leads to activation of the transcription factor NF-κB that increases expression of the caspase inhibitors cIAP1 and cIAP2. Hence, in most cells, apoptosis is induced by TNF-α only in combination with inhibitors of proteins and mRNA expression, such as cycloheximide or actinomycin D. TNFR2 lacks a death domain (DD) but has a cytoplasmic motif that binds to TRAF2 and signals to inflammation via activation of the NF-κB pathway. Furthermore, the binding of TNF-α to TNFR2 leads to the shedding of TNFR2, which now acts as a soluble receptor, thereby inhibiting TNF-α activity. The simultaneous engagement of both receptors (TNFR1 and TNFR2) amplifies TNF-α-induced apoptosis because TRAF2 recruits cIAP that, in turn, stimulates the degradation of TNFR1 and TRAF2 itself [14,17,50,53].

Cell death-induced by TNF-α in mammals is classified into apoptosis or necrosis based on morphological and biochemical criteria. Apoptosis is characterized by plasma membrane blebbing, exposure of phosphatidyl serine, cell shrinkage, condensation and margination of nuclear chromatin, degradation of DNA into nucleosomal units and formation of apoptotic bodies. However, the hallmark of an apoptotic process is its dependence on caspase activation [27,31].

Minimal or no nuclear changes are seen in cells dying by necrosis. Necrotic cells swell and collapse like a punctured balloon, spilling their contents in the medium, thereby precluding an inflammatory response. This mechanism is extremely important for activating the immune system. The swelling cells take up some dyes such as propidium iodide and trypan blue because of their leaky cell membrane. There is no detectable oligonucleosomal degradation of DNA, and this form of cell death is not accompanied by caspase activation. However, the cell destruction may be supported by the ATP and Ca^{2+} dependent activation of proteolytic enzymes such as cathepsins, calpains and proteasome [48]. Since necrosis is usually the consequence of metabolic disruption and ATP depletion, the ADP/ATP ratio has been used to differentiate apoptosis from necrosis [31].

A third mechanism by which cells may die is referred to as aponecrosis and involves a phenotype resulting from a more or less complete execution of the apoptotic pathway but with the outcome being necrosis [31]. Of the numerous downstream regulators capable of directing a cell towards a given type of cell death, at least six of them deserve special attention, and include (1) intracellular ATP, which provides the energy required for apoptosis, (2) reactive oxygen species (ROS), which are involved in necrosis [14], (3) BCL-2 protein, the mitochondrial antiapoptotic function of which can protect cells from necrosis [49], (4) intracellular Ca^{2+}, the levels of which are critical in determining entry into cell death, (5) the activation of proteolytic systems other than caspases, such those mediated by calpains, cathepsins and the proteasome [48], and (6) the release of mitochondrial apoptogenic factors such as endonuclease G and AIF (apoptosis-inducing factor), which cause DNA condensation and degradation, and Smac/Diablo and HtrA2/Omi, which facilitate the activation of caspases [27,31,42,48,50,53; see also other reviews in this issue].

The discovery in 1998 that the inhibition of caspase activity sensitized murine fibroblasts and some human tumor cell lines to the cytotoxic effects of TNF-α provided a new opportunity to increase our knowledge about the role of caspases in cellular functions, including cell death [30,34,51,52]. Since then, there has been a marked increase in the number of studies showing that caspases are required in a variety of physiological and non-apoptotic processes, including erythrocyte and keratinocyte differentiation, cell cycle regulation, skeletal muscle differentiation, cell migration and receptor internalization [2, 4,15,18,30,41]. Likewise, the number of adapters and effectors recruited to the heteromeric TNF receptor complexes and shown to be involved in the signaling

pathways of necrosis, apoptosis, cell survival and proliferation has grown considerably [17,50,53].

A link between the cell cycle and apoptosis has been suggested based on the finding that manipulation of the cell cycle may prevent or induce an apoptotic response, depending upon the cellular context [1,3,43,47]. In many cell lines, inhibition of the Fas receptor and the TNF receptor-mediated caspase signaling pathway shifts FADD-death domain-mediated apoptosis to cell death by necrosis, in a phenomenon referred to as necroptosis [5,8,47]. This fourth pathway of cell death is observed in the presence of caspase inhibition and may be a physiologically regulated process mediated by classic cell death receptors in various cell types [5,8,23,34]. Indeed, there is evidence that this type of cell death functions as a cellular backup mechanism to ensure the elimination of damaged cells in a wide range of human diseases, including myocardial infarction and acute and chronic neurodegeneration, when apoptosis is inhibited [8]. In the following discussion, we will evaluate the critical roles exerted by caspases and cell cycle-regulated proteins in the early and late steps of the TNF signaling pathways to apoptosis, necrosis, necroptosis and proliferation.

Exploring the signaling pathways in distinct types of TNF-α-induced cell death

Although TNF-α was initially identified by its ability to kill tumor cells, most tumor cells do not undergo apoptosis in response to this cytokine [14,39,44]. Three tumor cell lines have been extensively used as models to study the molecular mechanisms involved in TNF-induced cytotoxicity: (1) the highly sensitive clone 164 of WEHI cells, a fibrosarcoma cell line derived from a Balb/c mouse tumor induced by methylcholanthrene, (2) L929 cells, a fibroblast-like cell line derived from the connective tissue of a C3H mouse, and (3) HeLa cells, an epithelial-like cell line derived from a human cervical epithelioid carcinoma that is only sensitive when incubated in the presence of protein synthesis inhibitors [14,51,52].

We have shown [L.A.V. Cordeiro, Master's dissertation, University of São Paulo, São Paulo, Brazil, and S.C. Enns, Master's dissertation, University of São Paulo, São Paulo, Brazil] that WEHI-164 cells treated with TNF-α (1-10 ng/ml) initially display the morphological changes typical of apoptosis in mitochondria and then in the nucleus and cytoplasm (Fig. 1). The mitochondria appear swollen, with characteristic alterations that include a large, clear matrix space and few crests. Only cells in advanced stages of chromatin condensation develop blebs and plasma membrane lysis, which may lead to secondary necrosis. An essential step in TNF-α cytotoxicity in WEHI-164 cells is the activation of the caspase cascade, which initiates with caspase-8 followed by caspases -2, -3, -9 and -6. In particular, caspase-3 activity is markedly greater in these cells compared with five other cell lines investigated. These proteases have hundreds of nuclear and cytoplasmic protein targets, the cleavage of which produces the morphology characteristic of apoptosis. Most of the biochemical and morphological effects induced by TNF-α in WEHI-164 cells are inhibited by z-VAD-fmk,

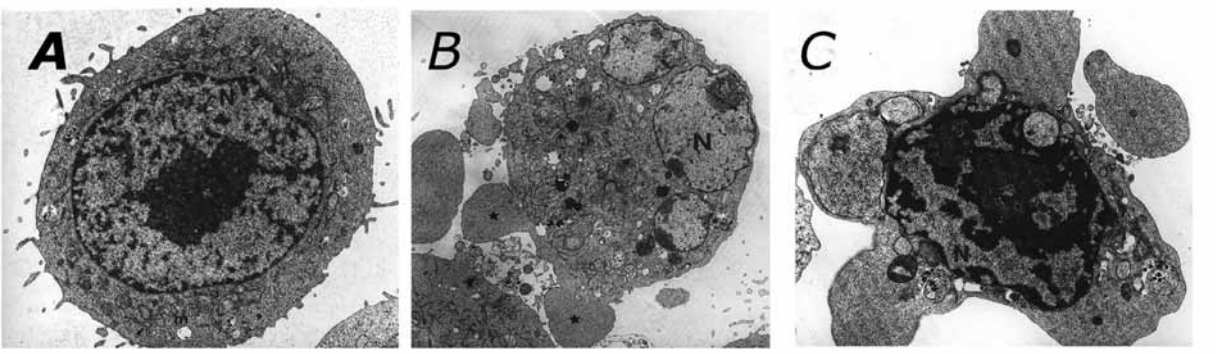

Figure 1. Sequence of ultrastructural changes associated with apoptosis induced by TNF-α (10 ng/ml) in WEHI-164 cells, a murine fibrosarcoma cell line. Panel **A** shows a cell with an intact cytoplasm and organelles. Panel **B** shows a cell in early apoptosis with extensive protrusion and blebbing of the plasma membrane, cytoplasmic shrinkage and nuclear fragmentation. Panel **C** shows a cell with chromatin condensation and compact granular masses around the nuclear membrane, as well as multiple apoptotic bodies in the cytoplasm. (**A**),(**B**) and (**C**) Bars = 2 μm.

z-IEDT-fmk and z-DEVD-fmk, three peptide-based caspase inhibitors [S.C. Enns, Master's dissertation, University of São Paulo, São Paulo, Brazil]. These findings indicated that WEHI-164 cells exposed to a low concentration of TNF display the morphological, biochemical and biological features of apoptosis (Fig. 1). However, at high concentrations (20-100 ng/ml), a small population of cells showed a phenotype typical of necrotic cell death [L.A.V. Cordeiro, Master's dissertation, University of São Paulo, São Paulo, Brazil].

A large number of cellular conditions and intrinsic factors that may contribute to the induction of apoptosis and necrosis by TNF-α in murine WEHI-164 and L929 cells, as well as in a few number human cell lines [14], have been identified. Distinct morphological and functional changes occur in the mitochondria of necrotic cells induced by TNF-α. However, the precise contribution of ROS [14,19,52], phospholipase A_2 [28], caspases [12,51,52], cathepsin D [9], the BCL-2 and BAX family of proteins [14,51,52], and cell cycle regulatory proteins [1,3,4,7,43,44] to the early, intermediate and late steps of mitochondria-dependent and independent cell death is very difficult to define at the molecular level.

TNF-α-induced mitochondrial ROS production and necrosis have been extensively studied in L929 cells [19,51,52]. The key molecular sensor that is oxidized by ROS and provokes cell lysis is unknown. However, when the caspase inhibitor z-VAD-fmk was used to counteract the TNF-α-induced signaling to necrosis, L929 cells unexpectedly became 1000-

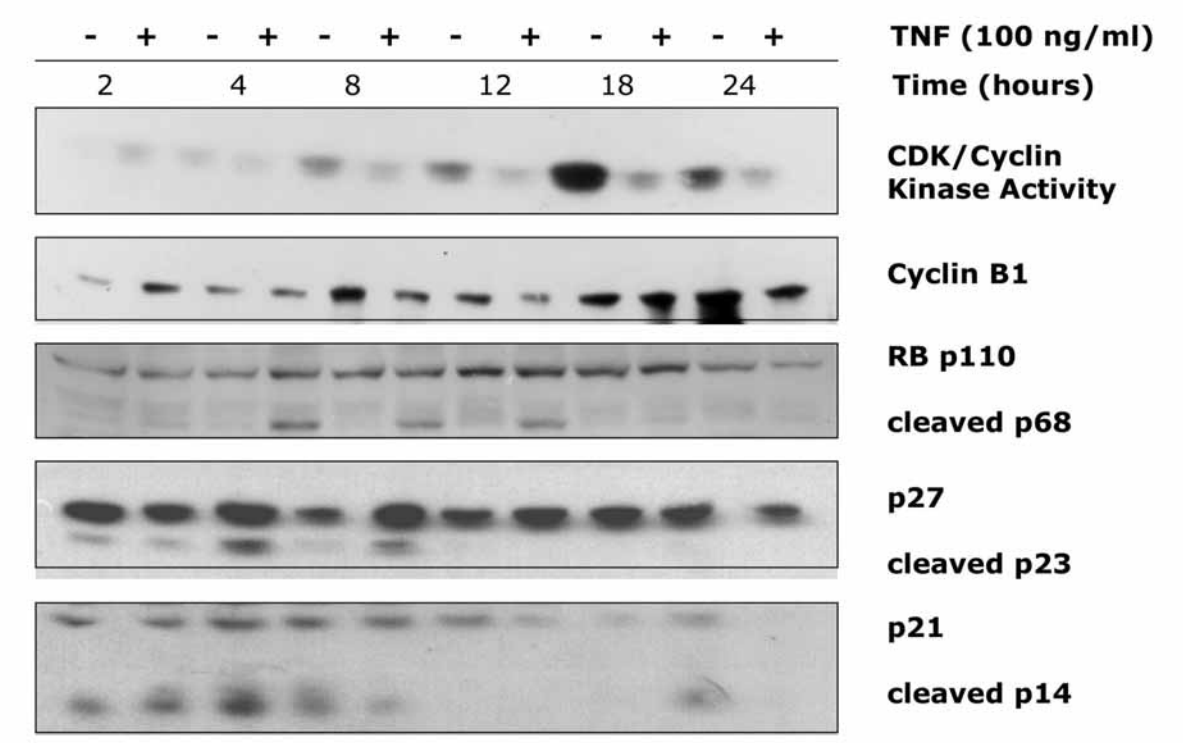

Figure 2. Variations in CDK-histone H1 kinase activity and in cyclin B1, RB, p27 and p21 in HeLa-S3 cells during progression from G_1 to the remaining stages of the cell cycle. Cells were first pre-incubated with lovastatin (50 μM) for 36 h and then with complete medium supplemented with fetal calf serum and 5000 μM mevalonate in the absence (-) or presence (+) of TNF-α (100 ng/ml) and harvested at the times indicated. For the CDK-histone H1 kinase activity experiments (**A**), cell lysates containing 10 μg of protein were incubated with histone H1 proteins in the presence of [γ-^{32}P]ATP and the radioabeled products were visualized by autoradiography after SDS-PAGE in 15% gels. For western blots (**B**), cell lysates containing 50-100 μg of protein were electrophoresed and transferred to a PVDF membrane. The membrane was incubated with monoclonal antibodies to human cyclin B1, RB, p27 and p21 (diluted 1:500; Calbiochem, Cambridge, MA), and developed by chemiluminescence (ECL detection method). Similar results were obtained in at least three additional independent experiments.

fold more sensitive to TNF-α [14,17,23,51,52]. The pre-treatment of Swiss NIH-3T3 fibroblasts, lymphoid U937 and Jurkat cell lines with the pancaspase peptide inhibitor z-VAD-fmk sensitized these cells to the necrotic effects of TNF-α [23,34,36]. Nevertheless, most of the apoptotic events induced by TNF-α in WEHI-164 and L929 cells were inhibited by z-VAD-fmk and z-IETD-fmk [S.C. Enns, Master's dissertation, University of São Paulo, São Paulo, Brazi]. Furthermore, apoptosis in WEHI-164 cells is a cell cycle regulated mechanism [12,13]. Indeed, only quiescent or G_1/G_o arrested WEHI-164 cells undergo necrosis, whereas apoptosis is induced in proliferating cells [12]. The differential processing of a caspase-8 isoform may be associated with the necrotic phenotype seen in WEHI-164 and L929 cells [12]. Based on these findings, a mechanism that involves a caspase-like enzyme-mediated surveillance and the removal of ROS-damaged mitochondria that can cause rapid necrosis has been proposed [14].

We have used Balb/c 3T3 murine fibroblasts (clone A31) as a model to explore the interaction of z-VAD-fmk and TNF-α in cell death. First, we demonstrated that TNF-α can act as fibroblast growth factor, depending on the culture conditions and the concentrations used, as previously described [44]. However, when exposed to TNF-α plus z-VAD-fmk, the cells stopped cycling and underwent cell death. Examination of these cells by transmission electron microscopy revealed a significant proportion with signs of apoptosis, despite a lack of caspase activity

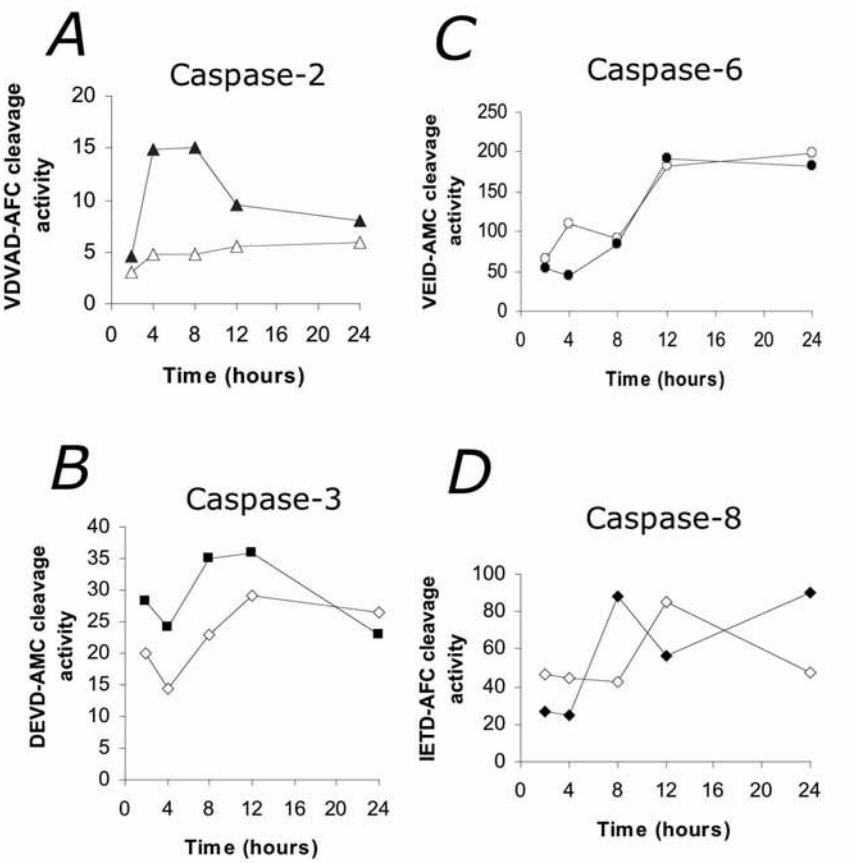

Figure 3. Activation of caspases in HeLa-S3 cells undergoing ordered cell cycle progression from G_1 to the subsequent stages of the cell cycle. The cells were pre-incubated with lovastatin (50 μM) for 36 h and then with complete medium supplemented with fetal calf serum and 5000 μM mevalonate in the absence (open) or presence (closed) of TNF-α (100 ng/ml) and harvested at the times indicated. The catalytic activity of caspase-2 (**A**), -3 (**B**), -6 (**C**) and -8 (**D**) was determined by monitoring the cleavage of the fluorogenic substrate by 50-100 μg of protein of cell lysate for 30-60 min. In some experiments, enzymatic activity was determined with a molar excess of peptide caspase inhibitor to assess the specificity of the enzyme being assayed. Similar profiles of activation were observed for each caspase in 3-4 additional experiments.

(Fig. 5). However, several morphological differences were identified in these cells. One important finding was the presence of intense cytoplasmic vacuolization (Fig. 5), despite a nuclear morphology that showed no obvious differences from typical apoptotic nuclei (Fig. 5C). Because of the potential involvement of ROS, we believe this cell death involved necrosis followed by apoptosis. The release of AIF and Endo G from mitochondria could account for the nuclear morphological changes characteristic of apoptosis [27]. Similar morphological changes have been seen in Swiss 3T3 fibroblasts [34], Jurkat lymphoma cells [8,36] and monocytic U937 cells [8]. We cannot exclude the possibility that the transition from apoptosis to necrosis could be dependent on a cell cycle stage such as described elsewhere [12,47]. Literature reports and our own data [1,3] support the idea that a commitment event that occurs at the interface of the G_1 and S phases is activated by caspases, resulting in cell death by apoptosis during the S and G_2/M phases. Consequently, this process can be specifically inhibited in the early G_1 phase [1]. In agreement with this conclusion, a recent study has shown that a cell population treated with z-VAD-fmk and TNF-α dies in the G_2/M phase via a necrotic-type cell death program [47]. The molecular mechanism involved in this caspase-independent cell death (necroptosis) may contribute to ischemic brain diseases [5,8,9,39]. A small molecule, necrostatin-1, that can inhibit the activation and autophagy of necroptotic cells has been identified [8].

TNF-α induces genes for cellular proliferation, resistance and apoptosis

Since 1985, various reports have shown that TNF-α exerts a mitogenic action in normal human fibroblasts, T lymphocytes and some transformed cells [39,44]. TNF-α acts synergistically with PDGF (platelet-derived growth factor, a competence factor) and EGF (epidermal growth factor, a progression factor) to ensure DNA synthesis in quiescent Balb/c 3T3 fibroblasts [39]. In the same cells, however, high concentrations of TNF-α (>10 ng/ml) were also cytotoxic. Cell death started 2 h after the addition of TNF-α and entered the S phase after approximately 12 h [39]. These results led to a search for immediate early genes and cell cycle-regulated genes, as well as for genes encoding protective proteins, during the TNF-α response. The first transcripts identified were of the transcription factors c-jun, junB, c-fos, c-myc and the NF-κB transcription factor subunits p105, p65 and pRelB [6,24,25]. Transcripts for p53 and p21 have also been identified in various tumor cells [10]. Additionally, most cells exposed to TNF-α express the genes for the oxygen free radical scavenging enzyme manganese superoxide dismutase, the zinc finger protein A20, and HSP-70 proteins [5,14,53]. These findings emphasized the complex nature of the TNF-α-induced mitogenic response.

Specific TNF-α effects on cell progression through G_1, S, G_2 and mitosis have been investigated using several approaches. Early studies indicated that entry into the S phase was necessary for cytotoxicity, whereas arrest in the G_1 phase increased the cellular resistance to TNF-α [1-3,7]. Cell cycle progression is dependent on the coordinated expression of the transcription factors c-myc and E_2F, which regulate the expression of cyclins and the activation of CDK/cyclin complexes [33,35]. Several lines of evidence indicate that the overexpression of *c-myc* and *cyclin D3* sensitizes cells to TNF-α cytotoxicity [20,25,26]. These early cell cycle genes are induced in normal fibroblasts and cancer cells *en route* to apoptosis under a variety of stimuli [40]. On the other hand, the expression of CKIs, p16, p21 and p27, which bind to and inhibit CDK/cyclin complexes and/or PCNA, causes cell cycle arrest, thereby, allowing more time for DNA repair or the blockade of cell death [33,35]. However, p21 has anti-apoptotic and proliferative functions under certain conditions and in specific systems [37,55,56].

To confirm the hypotheses of our previous studies [1-3] regarding cell cycle-specific effects of TNF-α, HeLa and WEHI-164 cells were G_1-phase synchronized by the lovastatin/mevalonic acid method [22] and expression and activity of cell cycle proteins examined [A.M. Sarr, Master's dissertation, University of São Paulo, São Paulo, Brazil, and V. Ferreira-Silva, Doctoral thesis, University of São Paulo, São Paulo, Brazil]. After the release of G_1 phase blockade, various alterations, including a time-dependent activation of CDK-histone H1 kinase activity, as well as cyclin B expression and pRB phosphorylation were observed in control HeLa cells (Fig. 2). These events characterized entry of the cells into the cell cycle. In parallel, time-matched HeLa cells treated under similar conditions with TNF-α showed a partial reduction in CDK-histone H1 kinase activity, whereas the expression of cyclin B did not change. In addition, TNF-α stimulated the

appearance of the cleaved forms of pRB, a protein substrate of caspases -3 and -7 [13,21]. In the interval in which control cells underwent the S phase (8-12 h), there was a slight increase in apoptosis (about 30%) in TNF-α-treated cells. Despite this delay, most cells (70%) continued the cell cycle normally after crossing the G_1/S checkpoint (Table 1). Hence, entry into or exit from the cell cycle is mediated by the threshold levels of cell cycle proteins and by the levels of survival and anti-apoptotic proteins, all of which contribute to determine the fate of the cell. Several studies support a role for the oncogenic transcription factors NF-κB, c-myc, p53 and E_2F in this intricate system that regulates the cell cycle and the checkpoints for entry into apoptosis [36,38].

Figure 3 shows the time courses for the full activation of caspases -2, -3, -6 and -8 in HeLa cells stimulated with TNF-α during the progression from G_1 phase to mitosis. At certain intervals, the levels of activity increased 100-200 fold in TNF-α-treated cells compared to the controls. Interestingly, the increase in caspase-6 activity paralleled the cell cycle progression. Compared to caspases -7 and -8, only the profile of mRNA expression for caspase-3 was upregulated by TNF-α treatment in a cell cycle-dependent manner. The pro-form of caspase-3 and its cleavage products, identified as p19 and p20 by western blotting, were present in the early hours of the cell cycle (Fig. 4). These findings support the suggestion that caspase activation is involved in the cell cycle. Indeed, various studies have provided evidence for the role of caspases in modulating the activation and inactivation of cell cycle proteins at specific checkpoints in G_1/S and G_2/M [33,35,38,55,56]. The cleavage of an inappropriate amount of cell cycle protein may culminate in apoptotic cell death. In agreement with this (and in contrast to human HeLa cells), murine WEHI-164 cells were unable to establish long-term cell cycle arrest in G_1 phase when treated with lovastatin,

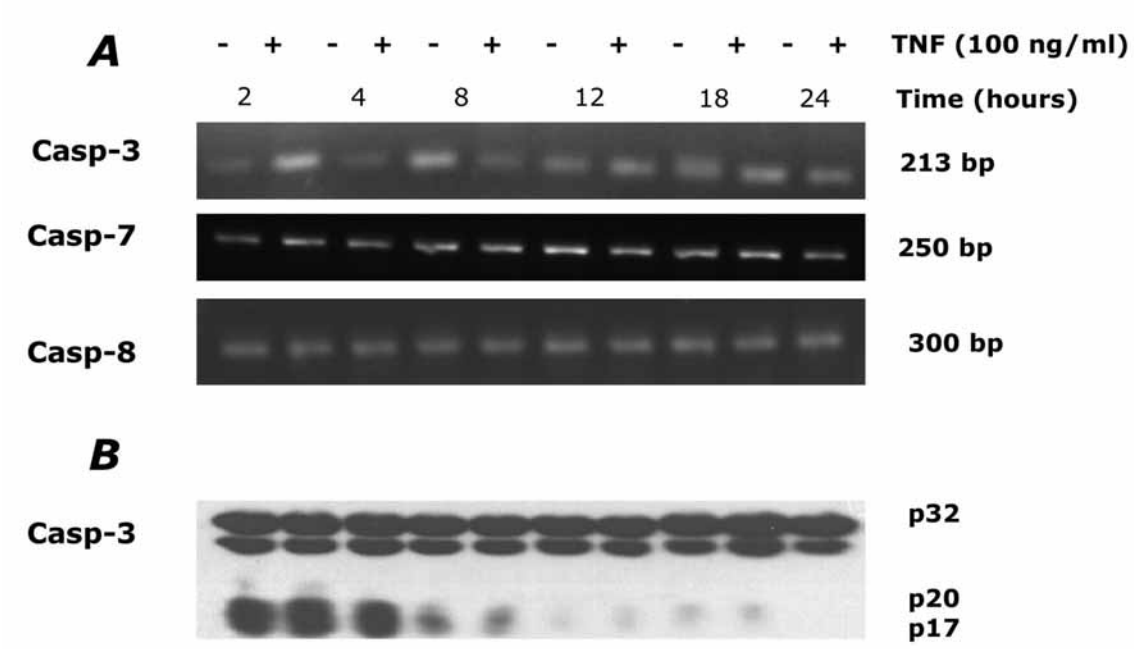

Figure 4. PCR analysis (**A**) for caspase -3, -7 and -8 and western blot analysis (**B**) for caspase-3 in HeLa cells during progression from G_1 to other phases of the cell cycle. The cells were pre-incubated with lovastatin (50 μM) for 36 h and then with complete medium supplemented with fetal calf serum and 5000 μM mevalonate in the absence (-) or presence (+) of TNF-α (100 ng/ml) and harvested at the times indicated. For the PCR experiments (**A**), 1 μg of total RNA extracted with Trizol was reversed-transcribed into cDNA and then amplified by PCR using pairs of specific primers. The PCR products were run in 2% agarose gels and detected by staining with ethidium bromide. For western blots (**B**), cell lysates containing 50-100 μg of total protein were electrophoresed and transferred to a PVDF membrane that was subsequently incubated with rabbit polyclonal antiserum to human caspase-3 (diluted 1:1000; Calbiochem, San Diego, Ca) and developed by chemiluminescence (ECL detection method). The presence of p19 and p20 cleaved products indicated caspase activation. Similar results were obtained in at least three additional independent experiments.

and most of them died within 12-20 h after G_1 phase synchronization. This increased sensitivity of WEHI-164 cells may originate from the higher caspase expression and basal activity in these cells [S.C. Enns, Master's dissertation, University of São Paulo, São Paulo, Brazil].

The cyclin-dependent kinase inhibitors p21 and p27 are well-known regulators of cellular differentiation and senescence [33,35]. The abundance and function of p21 and p27 are regulated by various mechanisms, including changes in transcription, translation, phosphorylation, ubiquitin-dependent and -independent proteasomal degradation, subcellular localization and cleavage by caspase [33]. The polyubiquitination of p21 and p27 and their subsequent degradation by the 26S proteasome is necessary for orderly progression through the cell cycle [33]. However, in cells undergoing uncontrolled progression, which results in cell cycle arrest or apoptosis, these proteins are selectively cleaved by caspase-3 [16,32,56].

Activated caspases -3, -6 and -8 are required for T and B cell activation and proliferation after mitogenic stimulation [11,38,55], and a comprehensive analysis of cell cycle-regulated genes in mouse fibroblasts and human HeLa cells using cDNA microarray analysis has provided evidence for periodical upregulation of caspase-3 mRNA in the G_1 phase of the cell cycle [54]. This observation is consistent with the data presented in Fig. 4. Woo *et al.* [55] reported that active caspase-3 specifically cleaved p21 associated with CDKs. This cleavage was required for the full activation of CDKs and assembly of the PCNA-CDK-cyclin complex and, ultimately, for cell cycling in mitogen-activated B cells [55]. Hence, a failure in p21 cleavage could account for the hyperproliferation of B and T lymphocytes in caspase-3-deficient mice [55]. A recent study has shown that caspase $3^{-/-}$ and caspase $7^{-/-}$ double-knockout mice die rapidly after birth and display exencephaly [29].

In proliferating human B cells stimulated by anti-CD40 and anti-CD180, caspase-6 and -8 activities are elevated for 12-48 h while caspase-3 activity is reduced after 12 h [38]. Treatment of these cells with the caspase-6 peptide inhibitor, z-VEID-fmk, inhibited the accumulation of cyclin D2, cyclin D3 and CDK4 mRNA, but did not affect the expression of the apoptosis inhibitor cIAP2, which inhibits caspases -3 and -7 [38]. Other studies have shown that p21 interacts with procaspase-3 by its N-terminal sequence and prevents caspase-3 activation [37,45,46]. The procaspase-3/p21 complex serves as a checkpoint until caspase activity reaches a threshold that determines p21 cleavage and cell proliferation [45,55].

Table 1. Frequencies of apoptotic cells and viable cells during the cell cycle phases G_1, S and G_2/M in HeLa-S3 cells after G_1 phase synchronization with lovastatin (Lov) and stimulation with mevalonate (Mev) in the absence or presence of TNF-α.

Treatment	Time (h)	Apoptosis	Cell cycle distribution (%)		
			G_1	S	G_2/M
Lov/Mev	2	15.6 ± 5.3	78.8 ± 2.4	16.0 ± 2.1	14.3 ± 5.9
	4	18.0 ± 6.2	77.6 ± 0.4	17.8 ± 2.6	16.6 ± 6.2
	8	33.5 ± 2.1*	77.4 ± 5.0	14.3 ± 1.9	12.4 ± 1.3
	12	17.8 ± 1.8	76.2 ± 1.0	19.5 ± 4.2	11.9 ± 3.9
	24	15.0 ± 7.1	60.4 ± 15.0*	31.7 ± 7.0*	20.8 ± 3.8
Lov-Mev-TNF-α					
	2	14.6 ± 0.1	80.1 ± 4.0	15.0 ± 2.8	14.3 ± 5.3
	4	15.5 ± 1.0	79.5 ± 1.3	15.9 ± 4.6	15.2 ± 6.4
	8	39.6 ± 4.5*	82.1 ± 1.0	17.8 ± 9.3	11.1 ± 4.4
	24	24.2 ± 0.2	76.4 ± 2.8	18.1 ± 1.1	9.1 ± 1.1*

HeLa-S3 cells were incubated with lovastatin (50 μM) for 36 h and then washed and incubated with complete medium supplemented with fetal calf serum and mevalonate (5000 μM) in the absence or presence of TNF-α (100 ng/ml). The cells were fixed in 70% ethanol and treated with RNAse (100 μg/ml) followed by staining with propidium iodide (50 μg/ml). The DNA content was determined by flow cytometry (FACS Star Beckton Dickinson). The cell profiles were analyzed using the CellQuest and ModFit softwares. The values are expressed as the mean ± standard deviation of three independent experiments**.** Statistical significance was determined with the paired Student's *t*-test. *$p<0.05$ when compared to the groups before or after the incubation time indicated.

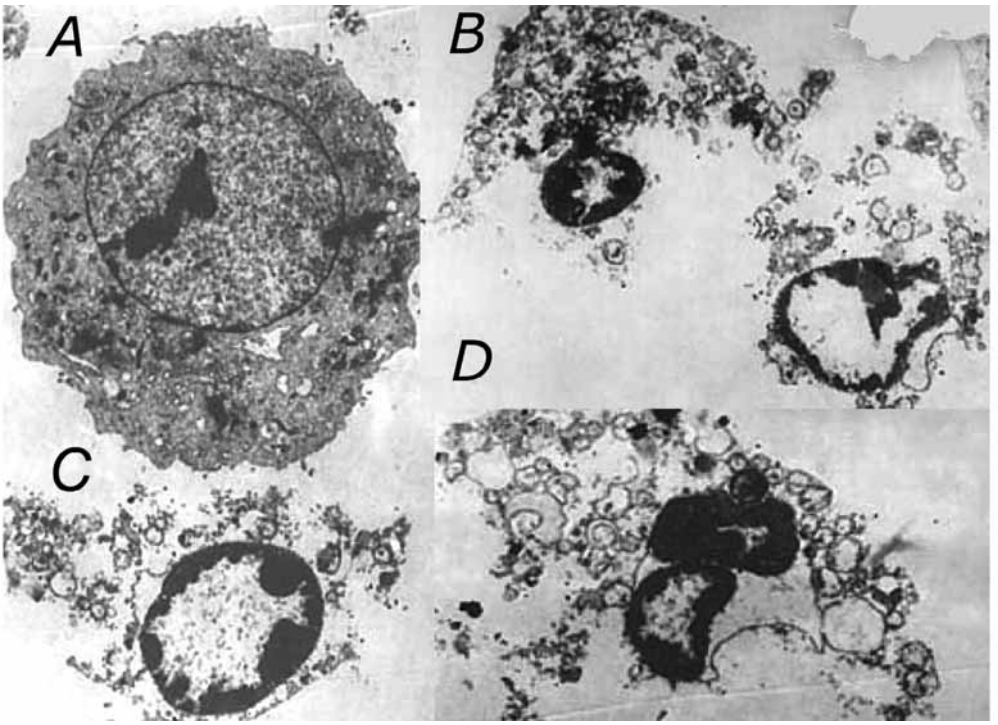

Figure 5. Ultrastructural changes associated with the necroptotic process induced by a combination of TNF-α (10 ng/ml) and z-VAD-fmk (20 μM) in NHI3T3 murine fibroblasts (a diploid cell line). Panel **A** shows a cell with intact organelles and plasma membrane, whereas **B**, **C** and **D** show cells with extensive cytoplasmic degeneration and plasma membrane rupture. Note that necroptotic cells show apoptosis-like chromatin condensation. This is particularly evident in the cells in panel **C**, which contain masses of condensed chromatin around the nuclear envelope. (**A**),(**B**), (**C**) and (**D**) Bars = 2 μm.

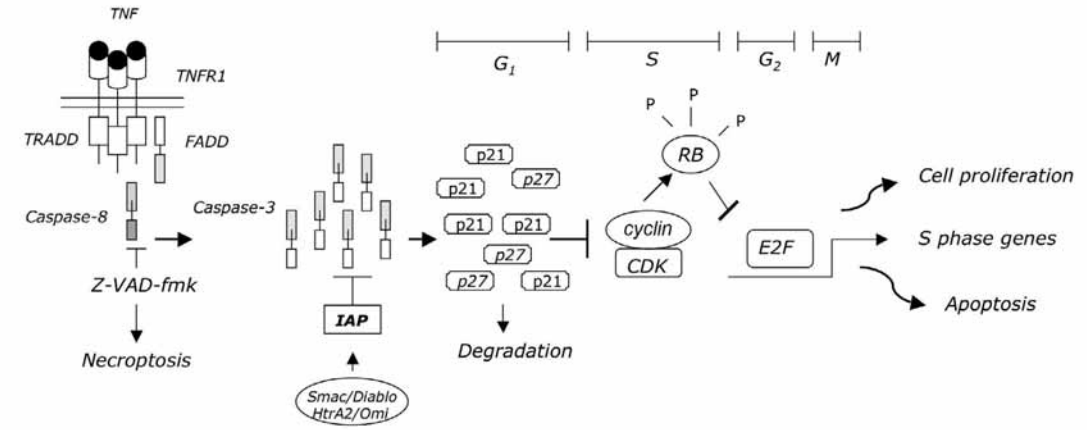

Figure 6. The critical steps in the TNF/TNF-receptor signaling pathway and cell cycle progression in which the regulated activation of caspases -8 and -3 and the cleavage of p21 and p27 can lead to apoptosis, and proliferation. In response to TNF receptor activation, a limited amount of caspase-8 and caspase-3 is activated. Caspase-3 cleaves p21 and p27, and facilitates full activation of the CDK/cyclin complex. The phosphorylation of RB by these CDK/cyclin kinases releases E2F, which then promotes the transcription of S phase genes. The critical step for apoptosis depends on how much caspase is activated and buffered by IAP proteins. Replication errors, DNA damage and oxidative stress activate a mitochondrial intrinsic pathway that causes the release of the mitochondrial factors Smac/Diablo and HtrA/OMI. Although the molecular mechanism is still not yet well understood, Smac/Diablo and HtrA/OMI could facilitate caspase activation by cleavage or by relieving the IAP-mediated inhibition of caspases. When the physiological threshold is crossed, caspases -8 and -3 and -7 initiate the cleavage of many important proteins involved in the cell cycle and in cytoskeletal and cellular functions, thereby triggering programed cell death. In cells treated with z-VAD-fmk, the absence of caspase activity promotes cell death by necroptosis.

In accordance with this idea, a recent study has shown that the treatment of NIH3T3 fibroblasts with a combination of TNF-α and z-VAD-fmk causes the overexpression (5-7 fold increase) and nuclear accumulation of p21 [56]. More importantly, these cells accumulate in the G_2/M phase and then die by necroptosis [36,47]. These findings provide further evidence for two decision scenarios. In the first of these, the ubiquitination of caspase-3 via the ubiquitin-protein ligase activity of cIAP2 and the destruction of this caspase by the 26S proteasome at critical times of the cell cycle could maintain caspase activity at minimal levels, thereby resulting in a proliferative response. In the second scenario, the IAP antagonists Smac/DIABLO and HtrA2/Omi released from mitochondria could overcome the inhibitory effect of IAP on caspase-3, thereby raising its activity. Consequently, the amplification of caspase activation and the attentuation of proteasomal degradation could direct cells to an apoptotic response [18]. Figure 3 shows the critical steps in TNF-α/TNF-receptor activation and the cell cycle machinery in which the regulated activation of caspases -8 and -3 and the cleavage of p21 and p27 can lead to apoptosis, necroptosis and proliferation.

CONCLUSIONS

Much work has been devoted to investigating the mode of action of members of the TNF-α/TNFR superfamily in order to identify the genes and proteins that are activated or inactivated in apoptotic and proliferative pathways. There is increasing evidence that caspases play an important role in establishing a threshold for apoptosis by controlling the degradation of key proteins involved in these two opposing cellular responses.

In this article, we have discussed the critical steps involved in the induction of apoptosis or entry into the cell cycle based on studies of HeLa-S3 cells synchronized in G_1 phase with lovastatin, and have considered the time-dependent analysis of the expression and activity of cell cycle proteins and caspases in response to TNF-α. The literature data support a view in which the entry into apoptosis is dependent on the combined actions of cyclin dependent kinases (CDKs), phosphatases and the abundance of mitogenic, survival and anti-apoptotic factors. Our data also suggest that this entry depends on a caspase activity threshold that is kept in check by IAP antagonists, but is fully amplified after the release of the mitochondrial factors Smac/Diablo and HtrA/OMI. Although the molecular mechanism remains unclear, Smac/Diablo and HtrA/OMI may facilitate caspase activation by relieving the inhibition imposed by IAP proteins on caspases. Since apoptosis occurs only when caspase activity passes a critical threshold, some tumor cells may undergo overt proteolytic destruction because of the overexpression of IAP caspase inhibitors or of a critical caspase protein substrate, the cleavage of which results in an irreversible commitment to cell death. Mutations in endogenous caspase substrates, particularly at caspase cleavage sites, could also account for enhanced protection towards apoptosis in cancer cells. Further studies are required to explore the association between tumor proliferation and the reduced or altered cleavage of caspase substrates during the cell cycle in tumor cells.

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REFERENCES

1. Belizário JE, Dinarello CA (1991) Interleukin-1, interleukin-6, tumor necrosis factor-α and transforming growth factor-β increase cell resistance to TNF cytotoxicity by growth arrest in the G_1 phase of the cell cycle. *Cancer Res*. **51**, 2379-2385.
2. Belizário JE, Lorite MJ, Tisdale MJ (2001) Cleavage of caspases -1, -3, -6, -8 and -9 substrates by proteases in skeletal muscle from mice undergoing cancer cachexia. *Br. J. Cancer* **84**, 1135-1140.
3. Belizário JE, Sherwood S, Beçak W (1999) Induction of apoptosis in cancer cells by TNF and butyrolactone, a chemical inhibitor of cyclin-dependent kinases. *Braz. J. Med. Biol. Res*. **32,** 473-482.
4. Belizário JE, Tilly J, Sherwood SW (1993) Caffeine potentiates the lethality of tumor necrosis factor in cancer cells. *Br. J. Cancer* **67**, 1229-1235.
5. Berghe TV, Loo GV, Saelens X, Gurp MV, Brouckaert G, Kalai M, Declercq W, Vandenabeele P (2004) Differential signaling to apoptotic and necrotic cell death by Fas-associated death domain protein FADD. *J. Biol. Chem*. **279**, 7925-7933.

6. Claudio S, Segade F, Wrobel K, Ramos S, Bravo R, Lazo P (1996) Molecular mechanisms of TNF-α cytotoxicity: activation of NF-κB and nuclear translocation. *Exp. Cell Res*. **224**, 63-71.
7. Darzynkiewicz Z, Williamson B, Carswell EA, Old J (1984) The cell cycle specific effects of tumor necrosis factor. *Cancer Res*. **44**, 83-90.
8. Degterev A, Huang Z, Boyce M, Li Y, Jagtap, Mizushima N, Cuny GD, Mitchison TJ, Moskowitz MA, Yuan J (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nature Chem. Biol*. **1**, 112-119.
9. Deiss LP, Galinka H, Berissi H, Cohen O, Kimchi A (1996) Cathepsin D protease mediates programmed cell death induced by interferon-γ, Fas/APO-1 and TNF-α. *EMBO J*. **15**, 3861-3870.
10. Donato NJ, Perez M (1998) Tumor necrosis factor-induced apoptosis stimulates p53 accumulation and p21/WAF1 proteolysis in ME-180 cells. *J. Biol. Chem*. **27**, 5067-5072.
11. Falk M, Ussat S, Reiling N, Wesch D, Kabelitz D, Adam-Klages S (2004) Caspase inhibition blocks human T cell proliferation by suppressing appropriate regulation of IL-2, CD25, and cell-cycle-associated proteins. *J. Immunol*. **173**, 5077-5085.
12. Faraco PR, Ledgerwood EC, Vandenabeele P, Prons JB, Bradley JR (1999) Tumor necrosis factor induces distinct patterns of caspase activation in WEHI-164 cells associated with apoptosis or necrosis depending on cell cycle stage. *Biochem. Biophys. Res. Commun.* **261**, 385-392.
13. Fattman CL, Scott MD, Dou QP, Johnson DE (2001) Sequential two-step cleavage of the retinoblastoma protein by caspase-3/-7 during ectoposide-induced apoptosis. *Oncogene* **20**, 2918-2926.
14. Fiers W, Beyaert R, Declercq W, Vandenabeele P (1999) More than one way to die: apoptosis, necrosis and reactive oxygen damage. *Oncogene* **18**, 7719-7730.
15. Garay-Malpartida JM, Occhiucci J, Alves J, Belizário JE (2005) CaSPredictor: a computer based tool for caspase substrate prediction. *Bioinformatics* **21**, 169-176.
16. Gervais JLM, Prem S, Zhang H (1998) Cleavage of CDK inhibitor p21/CIP1/WAF1 by caspases is an early event during DNA-induced apoptosis. *J. Biol. Chem*. **273**, 19207-19212.
17. Hehlgan T, Pfeffer K (2005) The intriguing biology of the tumor necrosis factor/tumor necrosis factor receptor superfamily: players, rules and the games. *Immunology* **115**, 1-20.
18. Henderson CJ, Aleo E, Fontanin A, Maestro R, Paroni G, Brancolini C (2005) Caspase activation and apoptosis in response to proteasome inhibitors. *Cell Death Differ.* **12,** 1240-1254.
19. Higuchi M, Aggarwal BB, Yeh ETH (1997) Activation of CPP32-like protease in tumor necrosis factor-induced apoptosis is dependent on mitochondrial function. *J. Clin. Invest*. **99**, 1751-1758.
20. Janicke RU, Lin XY, Lee FHH, Porter AG (1996) Cyclin D3 sensitizes tumor cells to TNF-induced, c-Myc dependent apoptosis. *Mol. Cell. Biol*. **16**, 5245-5253.
21. Janicke RU, Walker PA, Yu X, Porter AG (1996) Specific cleavage of the retinoblastoma protein by an ICE-like protease in apoptosis. *EMBO J*. **15**, 6969-6978.
22. Keyomarsi K, Sandoval L, Band V, Pardee AB (1991) Synchronization of tumor and normal cells from G_1 to multiple cell cycles by lovastatin. *Cancer Res*. **51**, 3602-3609.
23. Khwaja A, Tatton L (1999) Resistance to the cytotoxic effects of tumor necrosis factor α can be overcome by inhibition of FADD/caspase-dependent signaling pathway. *J. Biol. Chem*. **274**, 36817-36823.
24. Klefstrom J, Koskinen PJ, Saksela E, Jäättelä M, Bravo R, Alitalo K (1993) A sub-set of immediate early mRNAs induced by tumor necrosis factor-α during cellular cytotoxic and non-cytotoxic responses. *Int. J. Cancer* **55**, 655-659.
25. Klefstrom J, Vastrik I, Saksela E, Valle J, Eilers M, Alitalo K (1994) C-myc induces cellular susceptibility to the cytotoxic action of TNF. *EMBO J*. **13**, 5442-5450.
26. Kranenburg O, van der Eb AJ, Zantema A (1996) Cyclin D1 is an essential mediator of apoptotic neuronal cell death. *EMBO J*. **15**, 46-54.
27. Kroemer G, Martin SJ (2005) Caspas e-independent cell death. *Nature Med*. **11**, 725-730.
28. Kronke M, Adam-Klages A (2002) Role of caspases in TNF-mediated regulation of $cPLA_2$. *FEBS Lett*. **531**, 18-22.
29. Lakhani SA, Masud A, Kuida K, Porter Jr GA, Booth CJ, Mehal W, Inayat I, Flavell RA (2006) Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science* **311**, 847-851.
30. Launay S, Hermine O, Fontenay M, Kroemer G, Solary E, Garrido C (2005) Vital functions for lethal caspases. *Oncogene* **24**, 5137-5148.
31. Lemaster JJ (2004) Dying a thousand deaths: redundant pathways from different organelles to apoptosis and necrosis. *Gastroenterology* **129**, 351-360.
32. Levkau B, Koyama H, Raines EW, Clurman BE, Herren B, Orth K, Roberts JM, Ross R (1998) Cleavage of p21 and p27 mediates apoptosis in endothelial cells through activation of cdk2: role of a caspase cascade. *Mol. Cell Biol*. **1**, 553-563.
33. Lowe SW, Cepero E, Evan G (2004) Intrinsic tumor suppression. *Nature* **432**, 307-315.
34. Lüshen S, Ussat S, Scherer G, Kabelitz D, Klagest SA (2000) Sensitization to death receptor cytotoxicity by inhibition of Fas-associated death domain protein (FADD)/caspase signaling requirement of cell cycle progression. *J. Biol. Chem*. **275,** 24670-24678.
35. Massagué J (2004) G_1 cell-cycle control and cancer. *Nature* **432,** 298-306.

36. Matsumara H, Shimizu Y, Ohsawa Y, Kawahara A, Uchiyama Y, Nagata S (2000) Necrotic death pathway in Fas receptor signaling. *J. Cell Biol*. **151**, 1247-1255.
37. Nakamura K, Arai D, Fukuchi K (2004) Identification of the region required for the antiapoptotic function of the cyclin inhibitor p21. *Arch. Biochem. Biophys*. **431**, 47-54.
38. Olson NE, Graves JD, Shu GL, Ryan EJ, Clark EA (2003) Caspase activity is required for stimulated B lymphocytes to enter the cell cycle. *J. Immunol*. **170**, 6065-6072.
39. Palombella VJ, Vilcek J (1989) Mitogenic and cytotoxic actions of tumor necrosis factor in BALB/c 3T3 cells. *J. Biol. Chem*. **264**, 18128-18136.
40. Pandey S, Wang E (1995) Cells en route to apoptosis are characterized by the up-regulation of c-fos, c-myc, c-jun, cdc2 and RB phosphorylation, resembling events of early cell-cycle traverse. *J. Cell. Biochem*. **58**, 135-150.
41. Schwerk C, Schulze-Ostholf K (2003) Non-apoptotic functions of caspases in cellular proliferation and differentiation. *Biochem. Pharmacol*. **66**, 1453-1458.
42. Shi Y (2004) Caspase activation, inhibition, and reactivation: a mechanism view. *Prot. Sci.* **13**, 1979-1987.
43. Shih S-C, Stutman O (1996) Cell cycle-dependent tumor necrosis factor apoptosis. *Cancer Res*. **56**, 1591-1598.
44. Sugarman BJ, Aggarwal BB, Hass PE, Figary IE, Palladino MA, Shepard HM (1985) Recombinant human tumor necrosis factor-alpha: effects on proliferation of normal and transformed cells in vitro. *Science* **230**, 943-945.
45. Suzuki AY, Tsutomi K, Akahane T, Araki T, Miura M (1998) Resistance to Fas-mediated apoptosis: activation of caspase-3 is regulated by cell cycle regulator p21 and ILP gene family. *Oncogene* **17**, 931-940.
46. Suzuki A, Tsutomi Y, Yamamoto N, Shibutani T, Akahane K (1999) Mitochondrial regulation of cell death: mitochondria are essential for procaspase3-p21 complex formation to resist Fas-mediated cell death. *Mol. Cell. Biol*. **19**, 3842-3847.
47. Ussat S, Wener U-E, Kruse, M-L, Luschen S, Scherer G, Kabelitz D, Adam-Klages, S (2002) Upregulation of p21WAF/Cip1 precedes tumor necrosis factor-induced necrosis-like cell death. *Biochem. Biophys. Res. Commun*. **294**, 672-679.
48. Vandenabeele P, Orrenius S, Zhivotovsky (2005) Serine proteases and calpains fulfill important supporting roles in the apoptotic tragedy of the cellular opera. *Cell Death Differ*. **12**, 1219-1224.
49. Vander-Heiden MG, Thompson CB (1999) Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? *Nature Cell Biol*. **1**, E209-E216.
50. Varfolomeev EE, Ashkenazi A (2004) Tumor necrosis factor: an apoptosis junkie. *Cell* **116**, 491-497.
51. Vercammen D, Beyaert R, Denecker G, Goossens V, Loo GV, Declerq W, Grooten J, Fiers W, Vandenabeele P (1988) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by TNF. *J. Exp. Med***. 187**, 1477-1485.
52. Vercammen D, Brouckaert G, Denecker G, Craen MV, Declerq W, Fiers W, Vandenabeele P (1998) Dual signaling of the Fas receptor: initiation of both apoptotic and necrotic cell death pathways. *J. Exp. Med***. 188**, 919-930.
53. Wallach D, Varfolomeev NL, Malinin NL, Goltsev YV, Kovalenko AV, Boldin M, P (1999). Tumor necrosis factor and Fas signaling mechanisms. *Annu. Rev. Immunol***. 17**, 331-367.
54. Whitfield ML, Sherlock G, Saldanha AJ, Murray JI, Ball CA, Alexander KE, Matese JC, Perou CM, Hurt MM, Brown PO, Botstein D (2002) Identification of genes periodically expressed in the human cell cycle and their expression in tumors. *Mol. Biol. Cell*. **13**, 1977-2000.
55. Woo M, Hakem R, Furlonger C, Hakem A, Duncan GS, Sasaki T, Bouchard D, Lu L, Wu GE, Paige C, Mak TW (2003) Caspase-3 regulates cell cycle in B cells: a consequence of substrate specificity. *Nature Immunol*. **4**, 1016-1022.
56. Zhang Y, Fujita N, Tsuruo T (1999) Caspase-mediated cleavage of p21/WAF21/Cip1 converts cancer cells from growth arrest to undergoing apoptosis. *Oncogene* **18**, 1131-1138.

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