β-actin is required for mitochondria clustering and ROS generation in TNF-induced, caspase-independent cell death

Jinquan Li[1], Qinxi Li[1,*], Changchuan Xie[1], Huamin Zhou[1], Yuqian Wang[1], Na Zhang[1], Hanjuan Shao[1], Siu Chiu Chan[2], Xuanxian Peng[1], Sheng-Cai Lin[1,2] and Jiahuai Han[1,3,*]

[1]The Key Laboratory of the Ministry of Education for Cell Biology and Tumor Cell Engineering, School of Life Sciences, Xiamen University, Xiamen 361005, Fujian, China
[2]Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong
[3]Department of Immunology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
*Authors for correspondence (e-mail: jhan@scripps.edu, lqxmllsy@hotmail.com)

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Summary

Tumor necrosis factor (TNF)-α induces caspase-independent cell death in the fibrosarcoma cell line L929. This cell death has a necrotic phenotype and is dependent on production of reactive oxygen species (ROS) in the mitochondria. To identify genes involved in this TNF-induced, ROS-dependent cell death pathway, we utilized retrovirus insertion-mediated random mutagenesis to generate TNF-resistant L929 cell lines and we subsequently identified genes whose mutations are responsible for the TNF-resistant phenotype. In one such resistant line, β-actin was disrupted by viral insertion, and subsequent reconstitution of β-actin expression levels in the mutant line Actin^{mut} restored its sensitivity to TNF. Resistance to TNF in Actin^{mut} cells is signal specific since the sensitivity to other death stimuli is either unchanged or even increased. Comparable NF-κB activation and p38 phosphorylation in TNF-treated wild-type and Actin^{mut} cells also indicates that reduced expression of actin only selectively blocked some of the TNF-induced cellular changes. Actin cleavage involved in apoptosis does not occur in TNF-treated L929 cell death, as in HeLa cells. Consistent over-expression of a caspase-cleaved product, a 15 kDa actin fragment, had no effect on TNF-induced necrosis of L929 cell. By contrast, TNF-induced mitochondria clustering and ROS production were dramatically reduced in Actin^{mut} cells, indicating that actin-deficiency-mediated TNF resistance is most likely due to impaired mitochondrial responses to TNF stimulation. Our findings suggest that a full complement of actin is required for transduction of a cell death signal to mitochondria in TNF-treated L929 cells.

Key words: TNF, β-actin, Necrosis, ROS

Introduction

Tumor necrosis factor (TNF)-α is a pleiotropic cytokine that regulates numerous signaling pathways, such as those leading to the activation of phospholipases, mitogen-activated protein (MAP) kinases, NF-κB, apoptosis and necrosis (Beutler and Cerami, 1986; Liu and Han, 2001). It was originally identified as a factor that leads to hemorrhagic necrosis of an established tumor (Carswell et al., 1975; Old, 1985). In addition to its anti-tumor or anti-malignant cell effects, TNF has been shown to play a crucial role in the pathogenesis of acute and chronic inflammatory diseases (Beutler and Cerami, 1988). TNF-induced cell death can have apoptotic or necrotic phenotypes depending on the nature of the cell (Tewari and Dixit, 1996; Tewari and Dixit, 1995; Fiers et al., 1999; Beyaert and Fiers, 1994). The apoptosis can be inhibited by caspase inhibitor zVAD, whereas TNF-induced necrosis cannot be inhibited, but rather is enhanced, by zVAD treatment (Vercammen et al., 1998).

The effects of TNF on cells are mediated primarily through TNF receptor 1 (TNFR1, also termed TNF-R55, CD120a) (Boldin et al., 1996; Salvesen and Dixit, 1999; Green and Reed, 1998; Li and Yuan, 1999; Budihardjo et al., 1999). Upon binding to the receptor, TNF causes TNFR1 clustering and recruitment of TNFR1-associated death domain (TRADD) to the intracellular part of the receptor. TRADD subsequently recruits other effectors such as Fas-associated death domain (FADD), which is required for caspase-8 autoactivation in the caspase-dependent apoptosis pathway. The necrosis pathway might diverge somewhere downstream of these effectors since known pro-apoptotic caspases, or cytochrome *c* release, are not involved in this death pathway (Tartaglia et al., 1993; Vandevoorde et al., 1997; Fiers et al., 1999; Goossens et al., 1999; Vercammen et al., 1998). A recent study showed that FADD is not only required for apoptosis but is also required for necrosis (Lin et al., 2004). By contrast, receptor-interacting protein (RIP) and TNFR-associated factor 2 (TRAF2) in the TNFR1 complex are required for caspase-independent death (necrosis) but not for apoptosis (Lin et al., 2004; Holler et al., 2000). Despite the difference in the molecular mechanisms that control TNF-induced apoptosis and necrosis, mitochondria play a role in both types of cell death. Cytochrome *c* release from mitochondria is crucial for TNF-induced apoptosis but is absent in necrosis. Applying the free radical scavenger butylated hydroxyanisole (BHA) prevents L929 cells from

TNF-induced necrotic death (Goossens et al., 1995; Schulze-Osthoff et al., 1992; Brekke et al., 1992), but has no effect on TNF-induced apoptosis in PC60, KYM or MCF-7 cells (Fiers et al., 1999).

It has been increasingly recognized that reorganization of the cytoskeleton might exert important roles in both apoptosis and necrosis. In some cells, induction of apoptosis is accompanied by caspase-dependent β-actin cleavage to 15 and 31 kDa fragments (Mashima, et al., 1995; Mashima et al., 1997; Mashima et al., 1999; Cabado et al., 2003; Nakazono-Kusaba et al., 2002; Kayalar, et al., 1996). Ectopic expression of the 15 kDa actin fragment in HeLa and A431 cells has been shown to induce morphological changes resembling those in etoposide-induced apoptosis (Cabado et al., 2003). To date, there is no information regarding whether actin has a role in caspase-independent cell death. By contrast, a requirement of microtubules in caspase-independent death has been reported (De Vos et al., 1998; Vancompernolle et al., 2000). Translocation of mitochondria from dispersed distribution to clustering has been observed to be associated with TNF-induced apoptosis and necrosis (De Vos et al., 1998). Disrupting microtubules with nocodazole and inhibition of the motor protein kinesin can each block TNF-induced perinuclear redistribution of mitochondria in necrosis (De Vos et al., 1998). TNF induces persistent phosphorylation of oncoprotein 18 (Op18), a phosphorylation-responsive regulator of the microtubule dynamics (Vancompernolle et al., 2000). The hyperphosphorylation of Op18 promotes cell death. Since hyperphosphorylation retards the ability of Op18 to destabilize microtubules, the stabilization of the microtubule network is believed to be associated with TNF-induced necrosis (Vancompernolle et al., 2000).

In order to address further the mechanism of TNF-induced necrotic cell death, we have used a random gene disruption approach to generate a series of L929 mutants that are resistant to TNF-induced cell death, and have identified multiple functional genes involved in TNF-induced cell death (Wang and Han, 2000; Wang et al., 2001; Ono et al., 2001). A disrupted gene in one of the TNF-resistant lines was identified to encode β-actin. We have characterized this TNF-resistant line and explored the mechanism that underlies this resistance. It is shown here that β-actin appears to function downstream of TNFR1 and upstream of TNF-induced mitochondrial changes during TNF-induced necrosis in L929 cells. The function of actin in necrosis is regulated either by a signal from FADD, RIP and TRAF2, or an unknown signaling pathway that is initiated by a membrane-proximal region of TNFR1. Most strikingly, deficiency in the cellular contents of actin in the $Actin^{mut}$ cells causes severe defects in perinuclear redistribution of mitochondria and production of reactive oxygen species (ROS). Our present study has thus provided important evidence of the functional involvement and mechanism of actin in caspase-independent cell death.

Materials and Methods

Materials

Mouse TNF was obtained from Sigma. MitoTracker Green FM was obtained from Molecular Probes. Anti-actin C-terminal eleven residues antibody was from Sigma. zVAD was from Calbiochem. Anti-IκB-α was from Santa Cruz Biotechnology. Anti-phospho-p38 was from Cell Signaling. All cell lines were obtained from the American Type Culture Collection and cultured under the recommended conditions. Stable cell lines derived from L929 cells were selected by either G418 (1 mg/ml) or blasticidin S (10 μg/ml).

Vector construction, virus infection and plasmid transfection

The pDisrup retroviral vector was constructed using the MMLV retroviral vector pLNCX as a backbone (Miller and Buttimore, 1986), and standard recombinant DNA techniques were performed in its construction. The splicing donor and acceptor were designed according to human adenovirus type 2 major late mRNA sequence. Details of the plasmid as well as its construction are available upon request. Standard retrovirus production and cell infection procedures were used (Miller and Buttimore, 1986). β-actin cDNA was subcloned into expression vector pcDNA6 (Invitrogen) with a Blasticidin resistance gene. cDNA encoding a 15 kDa β-actin fragment was created by PCR and subcloned into pcDNA6 vector. SuperFect transfection reagent (Qiagen) was used for plasmid transfection in L929 cells.

Generating TNF-resistant mutants from L929 cells

In our experiments we used a L929 clone that exhibited a spontaneous survival rate of less than 1 in 10^6 after 48 hours of exposure to TNF at 100 ng/ml. We infected 5×10^6 cells with pDisrup virus and obtained ~10^4 G418-resistant clones. The G418-resistant clones and control parental L929 cells (cultured in parallel with G418-resistant clones but without infection with virus) were treated with TNF (100 ng/ml) for 48 hours. The re-grown clones were picked-up two weeks later and 23 TNF-resistant clones were obtained from the retrovirus-mutated cells, whereas no clone was recovered from the control parental L929 cells.

3′-RACE

Total RNA was isolated using Tri-Zol reagent (Invitrogen) and reverse transcription was performed with RT primer (5′-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA $GC(T)_{17}$-3′). A nested PCR was performed with RT product by using primers P1/Q1 (5′-ATG GGC TGA CCG CTT CCT-3′/5′-CCA GTG AGC AGA GTG ACG-3′) and P2/Q2 (5′-GAC GAG TTC TTC TGA CTA GCT AG-3′/5′-GAG GAC TCG AGC TCA AGC-3′), respectively. P1 and P2 are located on the *neo* resistance gene, whereas Q1 and Q2 are on the anchor sequence of QT. The PCR fragments were subcloned into the TA-cloning vector and sequenced.

Measurement of cell death

The cell death induced by TNF was determined by their plasma membrane integrity. The integrity of the plasma membrane was assessed by the ability of cells to exclude propidium iodide (PI; Sigma). Cells were trypsinized, collected by centrifugation, washed once with PBS, and resuspended in PBS containing 1 μg/ml PI. The level of PI incorporation was quantified by flow cytometry on a FACScan flow cytometer (Beckman coulter® EPICS XL™). The cell death induced by expression of TNFR1-associated proteins was determined by cotransfection of cells with various constructs in the presence of a green fluorescence protein (GFP) expression plasmid at a ratio of 4:1. Under these conditions, cells expressing GFP also expressed the cotransfected plasmid. The transfection efficiency was ~10%. The viability of cells was determined by the number of viable GFP expression cells (Tang et al., 2001; Liu et al., 1996).

Measurements of ROS

Generation of intracellular superoxide was determined according to

fluorescence of ethidium as a result of oxidation of hydroethidine [also known as dihydroethidium (HE); Molecular Probes]. 10 μg/μL HE in dimethyl sulphoxide (DMSO) was stored under nitrogen at 80°C. HE (6.6 μM) or a DMSO vehicle was added to cells at the same time and incubated for 15 minutes at 37°C. Fluorescence was measure by flow cytometry. Dichlorofluorescin-diacetate (DCFH-DA; Molecular Probes) fluoresces upon oxidation by hydrogen peroxide. DCFH-DA was stored at 4 mM in DMSO at –80°C. Cells were trypsinized, collected by centrifugation, washed once with PBS, and resuspended in PBS. DCFH-DA (20 μM) or a DMSO vehicle was added to cells at the same time. After 30 minutes, fluorescence was measure by flow cytometry on a FACScan flow cytometer.

Staining of mitochondria

Perinuclear redistribution of mitochondria was determined as described (De Vos et al., 1998). Briefly, cells were seeded in chambered coverslips and preincubated overnight. Mitochondria were stained with MitoTracker for 30 minutes at 37°C before analysis under fluorescence microscope.

Western blot analysis

Cell were lysed in lysis buffer (20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1 mM Na_3VO_4, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 40 mM β-glycerophosphate) containing 1% Triton X-100. 50 μg of protein was separated on a 12% SDS gel, transferred to a nitrocellulose membrane by electroblotting and processed for electrochemiluminescence (ECL) detection.

RNAi

Silencer™ short interfering (siRNA) cocktail kit (Ambion) was used to generate siRNAs. The siRNAs were prepared according to the manufacturer's instructions. The oligos used to generate the 500 bp DNA template for the dsRNA corresponding to the 3′-untranslated region of β-actin and to synthesize the dsRNA in vitro by T7 RNA polymerase were TTGGCGCTTTTGACTCAGGA and TGTAAG-GTAAGGTGTGCACT with and without T7 promoter sequence (TAATACGACTCACTATAGG) in the 5′ end. Lipofectamine 2000 (Invitrogen) was used to transfect siRNA into L929 cells. The transfection efficiency of siRNA in L929 cells was evaluated by rodamine-labeled siRNA against GFP (Qiagen) and was found to be close to 100%. The effective period of siRNA in L929 cells was determined by transfecting siRNA for GFP and then transfecting the GFP expression plasmid on day 1, 2, 3, 4 and 5, respectively. GFP expression was blocked on day 1, 2, 3 but not on day 4 and 5. Thus, multiple transfection of siRNAs for β-actin were performed on day 1, 4 and 7.

Results

Disruption of the β-actin gene in L929 cells confers resistance to TNF-induced necrosis

We first infected L929 cells with a retrovirus specially engineered to allow *neo* expression under the control of an active cellular gene upon integration. As described in our previous publications (Wang and Han, 2000; Wang et al., 2001; Ono et al., 2001), the retroviral vector was designed so that the *neo* gene was fused to the sequence of the exon located at the 3′ end of the viral insertion site. The identities of the disrupted genes in the various TNF-resistant cell lines were determined by 3′ rapid amplification of cDNA ends (RACE) of the fused *neo* mRNA.

The gene disrupted in two of the TNF-resistant cell clones was identified as β-actin. Because the 3′-RACE of fused *neo* mRNA yielded from these two clones are the same, we believe the two clones are originally from one cell and thus the same resistant line. A partial sequence of the fused gene product generated by retroviral insertion in this line (termed $Actin^{mut}$) is shown in Fig. 1A. The *neo* gene encoded by the viral vector was inserted at the 5′-untranslated region of β-actin and therefore disrupted one allele of the β-actin gene. Western blotting analysis revealed a reduced expression of β-actin (Fig. 1B), confirming that one active allele of the β-actin gene had been disrupted. $Actin^{mut}$ cells showed resistance to TNF-induced killing (Fig. 1C).

To establish whether the TNF resistance of $Actin^{mut}$ cells is genuinely due to the partial deficiency of β-actin expression, we stably transfected full-length cDNA encoding β-actin under the control of a cytomegalovirus (CMV) promoter or empty vector into the $Actin^{mut}$ cell line (designated $Actin^{mut}$+actin and $Actin^{mut}$+vector, respectively). Expression of β-actin in $Actin^{mut}$ cells restored sensitivity to TNF-induced cell death, whereas vector transfection of $Actin^{mut}$ cells had no effect on TNF sensitivity (Fig. 1D,E). The data shown in Fig. 1D and 1E were obtained using pools of transfectants; the same results were obtained when five individual clones of actin-transfected and vector-transfected cells were used (data not shown). To reproduce the finding that reduction of β-actin expression in L929 can lead to resistance to TNF-induced cell death, we used RNA interference (RNAi) to reduce β-actin expression. Because of the homology with α-actin in the coding region of mRNA, the 3′-untranslated region of β-actin was chosen as a targeting sequence for RNAi. To extend the time of RNAi treatment, we transfected L929 with siRNA every three days. As shown in Fig. 1F, on day 9 of RNAi treatment, the protein level of β-actin was reduced. In support of the role of β-actin in TNF-induced L929 cell death, the 9-day RNAi-treated L929 cells showed reduced cell death after TNF treatment (Fig. 1G). Collectively, our data demonstrate that the resistance to TNF-induced cell death observed in $Actin^{mut}$ cell lines is due to the reduced level of β-actin expression.

$Actin^{mut}$ cells are selectively resistant to TNF-induced necrosis

We then examined the sensitivity of $Actin^{mut}$ cells to several death stimuli to determine whether the β-actin-deficiency-caused death resistance is selective. As shown in Fig. 2A, $Actin^{mut}$ cells were resistant to TNF-induced cell death. By contrast, $Actin^{mut}$ was more sensitive to H_2O_2-induced and UV-induced cell death. The sensitivity of $Actin^{mut}$ to mitomycin-, vincristine- and fluoraouracil-induced cell death was comparable with that observed in the wild-type L929 cells. Thus, β-actin-deficiency-mediated TNF resistance is not due to a general promotion of cell survival, but is a selective impairment of a death pathway(s) used by TNF.

TNF-induced cell death in L929 cells is not promoted by caspases and can be enhanced by treatment with the pan-caspase inhibitor zVAD (Vercammen et al., 1998). We examined if this property is retained in $Actin^{mut}$ cells by treating them with TNF in the presence of different doses of zVAD. As shown in Fig. 2B, zVAD enhanced TNF-induced cell killing in both wild-type and $Actin^{mut}$ cells.

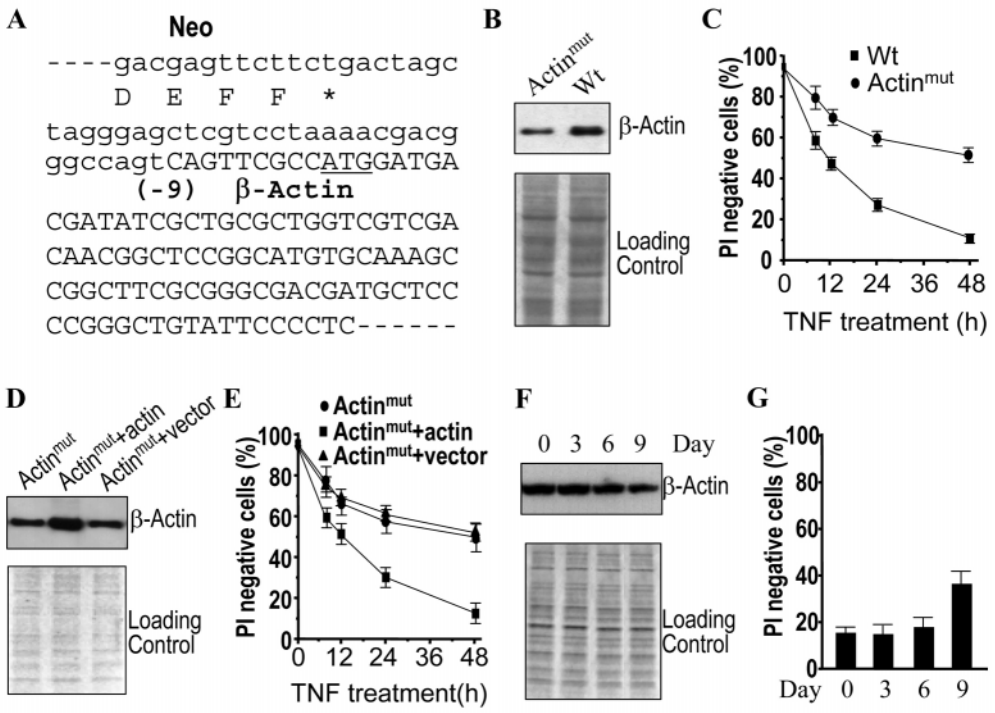

Fig. 1. β-actin mutation leads to a resistance of TNF-induced cell death in L929 cells. (A) The fused mRNA of *neo* and an endogenous gene in a TNF-resistant L929 clonal cell line was amplified by 3′-RACE. The junction sequence of the fused cDNA is shown, which reveals that the viral insertion occurred 5′ to the coding region of the β-actin gene. The amino acid sequence at the C terminus of *neo* is shown beneath the cDNA sequence. The sequence introduced by viral vectors is shown in lowercase. The number in parentheses indicates the position relative to the start codon of β-actin. (B) β-actin protein is reduced in β-actin mutant cells (Actin^{mut}). Western blotting was performed using anti-β-actin antibody. Equal loading of total cell lysates was determined by staining an identical SDS-PAGE with Coomassie Blue and a portion of the picture is shown. (C) Wild-type and Actin^{mut} cells were treated with TNF (100 ng/ml) for different periods of time and cell viability was assessed by propidium iodide (PI) exclusion. Results represent the means±s.e. (*n*=3). (D) Stable cell lines were generated from Actin^{mut} cells by transfection of β-actin expression vector (Actin^{mut}+actin) or empty vector (Actin^{mut}+vector). The expression level of β-actin was determined as in (B). (E) The sensitivity to TNF-induced cell death in Actin^{mut}, Actin^{mut}+actin and Actin^{mut}+vector was measured as in (C). (F) Parental wild-type L929 cells were treated with siRNA for β-actin as described in Materials and Methods for different periods of time as indicated. The level of β-actin protein was determined as in (B). (G) The viability of the RNAi-treated L929 cells was measured as in (C) after 48 hours TNF treatment.

β-actin deficiency impairs signaling to cell death but not to NF-κB or p38 activation

TNF-induced L929 cell death is initiated by TNFR1. A recent study suggested that FADD, TRAF2 and RIP are required for TNF-induced necrosis (Lin et al., 2004; Holler et al., 2000). It is also known that over-expression of some of these signaling proteins can kill cells (Liu et al., 1996). We examined whether β-actin deficiency had any effect on cellular changes mediated by over-expression of FADD, TRAF2 or RIP. We transiently expressed the TNFR1 cytosolic domain [TNFR1(CD)], TRADD, FADD, RIP or TRAF2 in wild-type and Actin^{mut} cells. GFP was co-expressed to select for the transfected cells and the viability of GFP-positive cells was determined. Actin^{mut} cells showed resistance to TNFR1(CD)-, TRADD- and FADD-induced cell death (Fig. 3A). Although RIP and TRAF2 are required for TNF-induced cell death in L929 cells, RIP and TRAF2 over-expression did not lead to significant cell death in either the wild-type or mutant cells (Fig. 3A). Since Actin^{mut} cells are resistant to TNFR1(CD)-, TRADD- and FADD-induced cell death, the lack of a full complement of cellular β-actin somehow fails to relay death signaling downstream of TRADD and FADD. β-actin is then either regulated by TRADD- or FADD-mediated signaling or is required for signal transduction downstream of TRADD and FADD.

TNF activates several intracellular signaling pathways including the NF-κB and p38 MAP kinase pathways (Liu and Han, 2001). RIP and TRAF2 are known to be essential for TNF-induced NF-κB and p38 activation. Because actin-binding protein or actin-containing structures were shown to interact with signaling molecules such as TRAF2 and p65/RelA (Leonardi et al., 2000; Are et al., 2000; Melamed et al., 1995), we examined whether TNF-induced NF-κB activation and p38 MAP kinase phosphorylation were affected by actin allelic inactivation. As shown in Fig. 3B, TNF-induced IκB degradation was comparable in Actin^{mut} and wild-type L929 cells, and TNF-induced p38 phosphorylation was also similar in wild-type and mutant cells. These data suggest that actin does not directly affect RIP, TRAF2 or their complexes. β-actin mutation impaired the TNF-activated death pathway but not all signaling pathways that are initiated by TNFR1.

The role of β-actin in TNF-induced necrosis is not related to actin cleavage reported in apoptosis

β-actin is cleaved by caspase-3 in some cells during the development of apoptosis (Mashima et al., 1995; Mashima et al., 1997; Cabado et al., 2003; Nakazono-Kusaba et al., 2002; Kayalar, et al., 1996). The 15 kDa C-terminal fragment of β-actin can be N-myristoylated and targeted to the mitochondria (Utsumi et al., 2003) and the generation of the 15 kDa fragment is believed to play a positive role in apoptosis (Cabado et al., 2003). We were able to reproduce the published data that etoposide treatment resulted in β-actin cleavage in HeLa cells, but did not detect any β-actin cleavage in TNF-treated L929 cells (Fig. 4A). This is consistent with published data that TNF-induced cell death in L929 cells is not associated with caspase

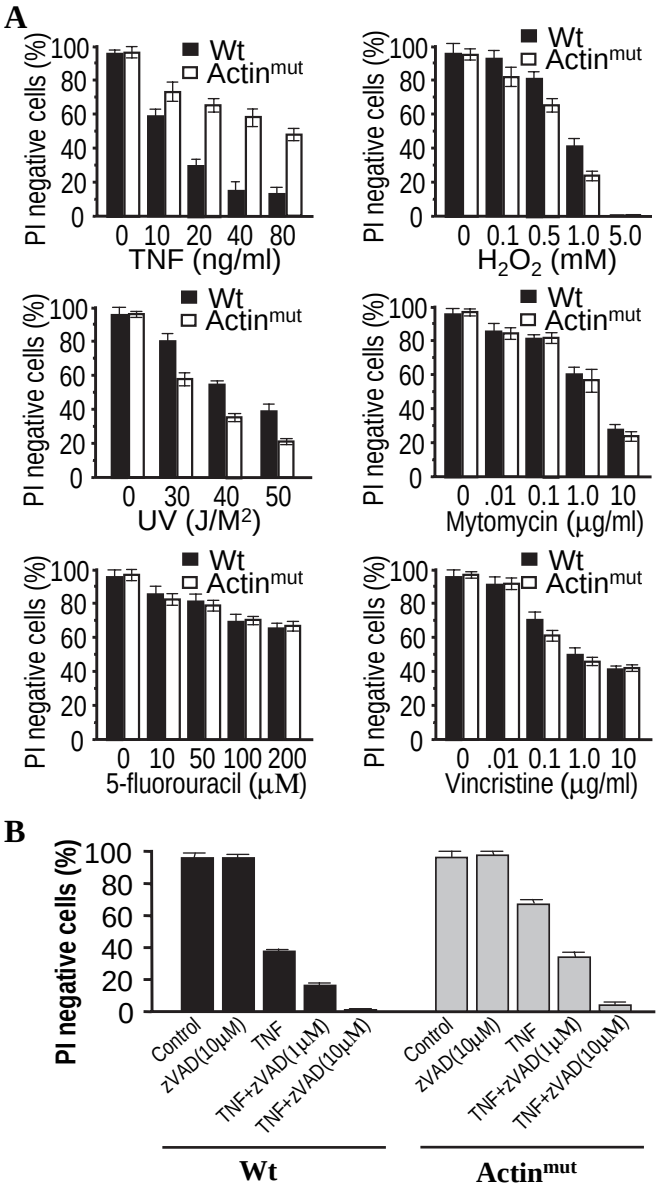

Fig. 2. Actin^{mut} cells are selectively resistant to TNF-induced killing. (A) Wild-type and Actin^{mut} cells were treated with indicated doses of TNF for 48 hours, H_2O_2 for 9 hours, UV, mytomycin for 96 hours, 5-fluorouracil for 65 hours and vincristine for 96 hours. Cell viability was measured. (B) Wild-type and Actin^{mut} cells were treated with TNF (100 ng/ml) and/or zVAD (1 μM or 10 μM) in different combinations for 18 hours. Cell viability was measured. Results represent the means±s.e. (n=3).

activation. We nevertheless evaluated whether the 15 kDa β-actin fragment has any role in TNF-induced cell death in L929 cells, through the stable transfection of an expression vector of the 15 kDa fragment in wild-type and Actin^{mut} cells. Expression of the 15 kDa protein was detected by Western blotting in pools of the stable transfectants (Fig. 4B). Expression of the 15 kDa protein did not affect sensitivity to TNF-induced cell death in wild-type or Actin^{mut} cells (Fig. 4C). The same result was obtained when five individual clones of transfectants were used (data not shown). These results indicate that cleavage of β-actin is not required for TNF-induced L929 cell death.

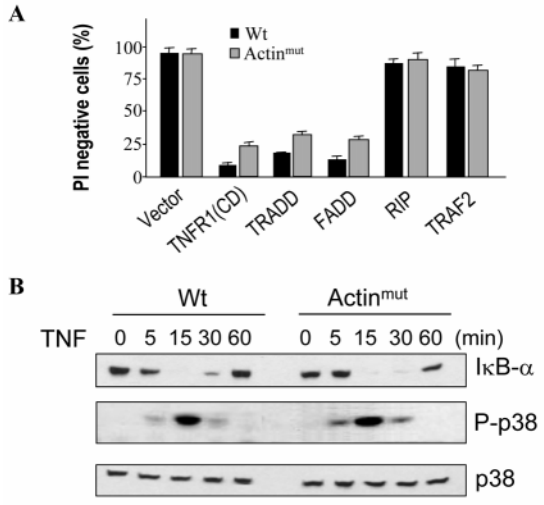

Fig. 3. Actin mutation interferes with the TNF-induced death signal but not with TNF-induced NF-κB and p38 activation. (A) Wild-type and Actin^{mut} cells were transiently transfected with the GFP expression vector and an expression vector of TNFR1(CD), TRADD, FADD, RIP or TRAF2. The death of the transfected cells (GFP positive) was calculated by counting sixteen random areas, and the differences in cell death were statistically significant (P<0.05, Student's t test). Three separate experiments were carried out with comparable results. (B) Wild-type and Actin^{mut} cells were treated with TNF (100 ng/ml) for different time periods as indicated. IκB-α and phospho (P)-p38 levels were determined by Western blotting. Equal loading of total cell lyates is shown by p38 protein levels.

β-actin deficiency impairs TNF-induced mitochondria clustering and ROS production

Mitochondria dysfunction plays a crucial role in TNF-induced L929 cell death (Goossens et al., 1995). It is known that perinuclear redistribution of mitochondria associates with TNF-induced cell death and precedes mitochondrial ROS production and cell death (De Vos et al., 1998). We used MitoTracker, a dye that is retained in the mitochondria by the thiol reactivity of its chloromethyl moiety, to monitor mitochondria location in TNF-treated wild-type and Actin^{mut} cells. Approximate 500 cells were analyzed in sixteen randomly chosen fields. As reported, the spatial redistribution of mitochondria in the majority of wild-type L929 cells changed from an originally scattered, bipolar or nearly symmetric distribution to an asymmetric, clustered distribution within one hour of TNF treatment (Fig. 5A). By contrast, TNF treatment did not lead to significant perinuclear redistribution of mitochondria in Actin^{mut} cells, even after longer treatment (up to 8 hours) with TNF (Fig. 5A, and data not shown). Given that microtubule stabilization is induced by TNF and is required for perinuclear redistribution of mitochondria (Vancompernolle et al., 2000), it is interesting to note that the two crucial components of the cytoskeleton – microtubule and actin – might cooperate with each other in TNF-induced mitochondria redistribution.

We next examined ROS production in TNF-treated Actin^{mut} cells in comparison with wild-type L929 cells. HE is often used to measure intracellular O_2^- because HE is oxidized to the fluorescent ethidium by O_2^-. The compound dichlorofluorescin-diacetate (DCFH-DA) is widely used to

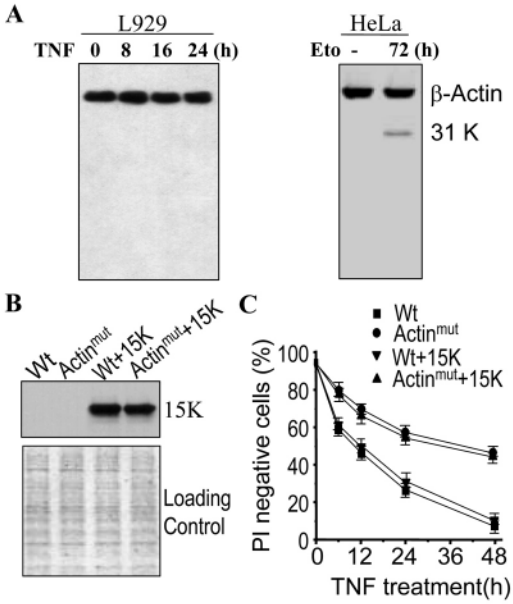

Fig. 4. Actin cleavage is not involved in TNF-induced L929 cell death. (A) L929 or HeLa cells were treated with TNF (100 ng/ml) or etoposide (Eto 40 μg/ml) for times as indicated. Actin and the 31 kDa fragment of actin were detected with the anti-actin polyclonal antibody against the C-terminal 11 residues. (B) Stable cell lines were generated from wild-type and Actin^{mut} cells by transfection of an expression vector of flag-tagged 15 kDa β-actin fragment (Wt+15K and Actin^{mut}+15K). The expression of the 15 kDa fragment was detected with anti-flag antibody M2. (C) The sensitivity to TNF-induced cell death in Wt, Actin^{mut}, Wt+15K and Actin^{mut}+15K was measured with propidium iodide (PI) exclusion. Results represent the means±s.e. (n=3).

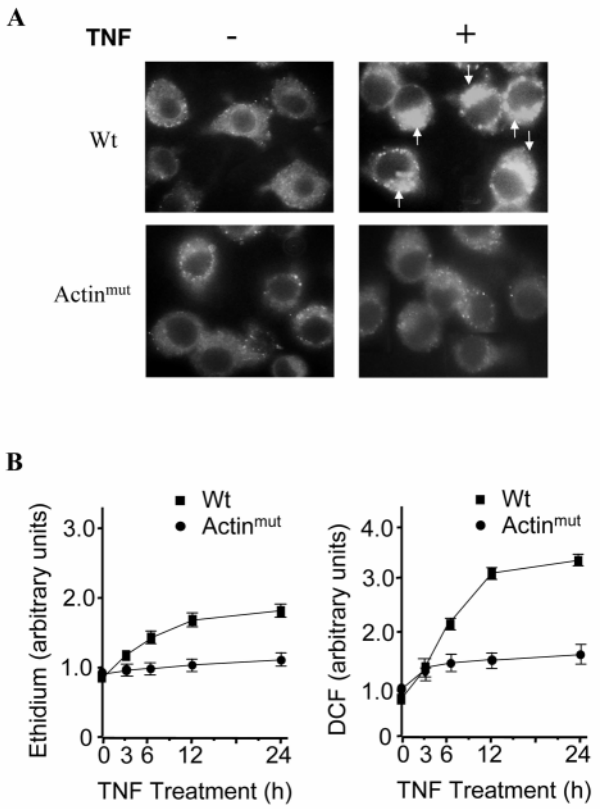

Fig. 5. Actin deficiency blocks TNF-induced perinuclear redistribution of mitochondria and ROS production. (A) Mitochondria of wild-type and Actin^{mut} cells were stained with MitoTracker, and the distribution of mitochondria was analyzed under fluorescence microscopy (×100). (B) Wild-type and Actin^{mut} cells were stained with hydroethidine (HE) or dichlorofluorescein-diacetate (DCFH-DA) together with PI at different times of TNF treatment. Levels of ethidium, the oxidation product of HE, or dichlorofluorescein (DCF), the fluorescent product of DCFH-DA, in PI-negative cells (live cells) were determined and shown. Results represent the means±s.e. (n=3).

measure the redox state of a cell. DCFH-DA is cell permeable and nonfluorescent. Cellular esterases cleave DCFH-DA to 2′-7′-dichlorodihydrofluorescein ($DCFH_2$). Peroxidases, cytochrome *c* and Fe^{2+} can all oxidize $DCFH_2$ to fluorescent 2′-7′-dichlorofluorescein (DCF) in the presence of H_2O_2, and thus DCF indicates H_2O_2 levels and peroxidase activity in the cell. We used HE and DCFH-DA to measure the redox state of L929 and Actin^{mut} cells. As shown in Fig. 5B, conversion of HE to ethidium or DCFH-DA to DCF increased in a time-dependent manner in TNF-treated wild-type cells, congruent with a previous report that TNF induces ROS production. Interestingly, the induction of ROS was significantly inhibited in Actin^{mut} cells. Since ROS induction is required for TNF-induced L929 cell death, β-actin-deficiency-mediated resistance to TNF-induced cell killing most likely resulted from a blocking of TNF-induced ROS production.

Discussion

Using a genetic mutational approach, we found that reduction of β-actin level caused resistance to TNF-induced necrosis in L929 cells (Fig. 1). The β-actin deficiency in L929 impairs TNF-induced mitochondrial changes and results in a decrease in cell death (Figs 5 and 1, respectively). An involvement of actin, actin filament or actin cytoskeleton in cell death processes has been suggested because of the morphological changes associated with the development of cell death (Mills, et al., 1999; Veselska et al., 2003; Melamed and Gelfand, 1999). A possible requirement of actin in cell death has been reported by several studies; however, many of the studies were based on chemical inhibitors that modulate the actin dynamics (Yamazaki et al., 2000; Kim et al., 2002; Cabado et al., 2003; White et al., 2001; Suria et al., 1999; Celeste Morley et al., 2003; Posey and Bierer, 1999; Wachter et al., 2003). Here, for the first time, we show genetic evidence of a requirement of actin in cell death. This is also the first report that points to the role of β-actin in necrotic cell death. Our data further suggest the notion that actin is not only involved in morphological changes that occur at late stages of cell death, but might also participate in transducing cell death signals.

Although there is no previous report regarding the involvement of actin or actin-related protein or structures in caspase-independent cell death (or necrosis), many lines of evidence have suggested a role of actin in signaling to apoptosis (Yamazaki et al., 2000; Kim et al., 2002; Cabado et al., 2003; White et al., 2001; Suria et al., 1999; Bando et al., 2002). Translocation of the actin-binding protein cofilin and cleavage fragments of actin and gelsolin to mitochondria has

been reported to promote cell death during the initiation phase of apoptosis (Chua et al., 2003; Kothakota et al., 1997; Kook et al., 2003; Cabado et al., 2003; Utsumi et al., 2003). But actin cannot be involved in the above events in TNF-treated L929 cells since caspases are not activated. We showed that the NF-κB pathway, a major survival pathway in TNF-treated cells (Mitchell, 2002; Tang et al., 2001), was not affected in Actin^{mut} cells (Fig. 3B), and that Actin^{mut} cells are not resistant to all cell death stimuli (Fig. 2A), but we cannot exclude the possibility that actin deficiency enhanced an unknown survival pathway. Since actin is involved in many cellular activities, the effect of actin deficiency on TNF-induced cell death could be a consequence of multiple events.

TNF-induced modification of the spatial redistribution of mitochondria occurs in both apoptosis and necrosis (De Vos et al., 1998). Microtubule networks are thought to be required for perinuclear redistribution of mitochondria. The motor protein kinesin was suggested to play a role in the movement of mitochondria (De Vos et al., 1998), and De Vos et al. showed that the membrane-proximal region of TNFR1 initiates the signaling to mitochondria clustering (De Vos et al., 1998). Since actin deficiency inhibited TNF-induced mitochondria clustering, we believe that the membrane-proximal region of TNFR1 might signal first to β-actin or actin filaments, which in turn trigger mitochondrial redistribution and clustering. It is most likely that not only microtubules but also the whole cytoskeleton is required for TNF-induced perinuclear redistribution of mitochondria. Vancompernolle and colleagues showed that TNF-induced changes in microtubule networks can be attributed to the TNF-induced phosphorylation of Op18 (Vancompernolle et al., 2000). The question as to whether rearrangement of actin filaments has a role in perinuclear redistribution of mitochondria or cell death remains unresolved because the commonly used actin microfilament toxin cytochalasin D and microfilament-stabilizing agents like jasplakinolide can affect many cellular events, making it impossible to assess their direct effect on mitochondria and mitochondria-mediated cell death.

TNF-induced cell death depends on the production of ROS by mitochondria (Goossens et al., 1995; Schulze-Osthoff et al., 1992; Brekke et al., 1992). A drastic reduction in induction of ROS in TNF-treated Actin^{mut} cells suggested that the decrease in cell death of Actin^{mut} cells is due to impaired TNF-induced ROS production. How actin is involved in TNF-induced ROS production is unclear at present. It is possible that perinuclear redistribution of mitochondria is required for ROS induction and the reduced ROS production in Actin^{mut} cells resulted from a blocking of mitochondrial movement. Although microtubules were shown to be required for perinuclear redistribution of mitochondria in TNF-treated cells, there is no evidence suggesting microtubules are required for ROS production. Thus, it is also possible that inhibition of mitochondria redistribution in the cell and ROS production are independent events, and that actin is targeted by the two signals or that actin is a diverging point targeted by the same signal to regulate the two independent processes. The TNF-induced actin response might be involved in signal transduction for ROS production in mitochondria or might directly take part in the machinery of ROS production. The pan-caspase inhibitor zVAD does not have any effect on perinuclear redistribution of mitochondria but dramatically enhances TNF-induced ROS production (De Vos et al., 1998), suggesting that ROS production is a later or separated event of perinuclear redistribution of mitochondria.

Studies using knockout cells revealed that FADD, TRAF2 and RIP are all required for TNF-induced ROS production and necrosis (Lin et al., 2004; Holler et al., 2000). Here, we show that there is little ROS induction by TNF in Actin^{mut} cells (Fig. 5B). However, whether actin receives signaling from FADD, TRAF2 and RIP is still an open question. Mitochondrial clustering seems to be independent from these effectors (De Vos et al., 1998); thus, actin might be regulated by an unknown signaling pathway that is initiated by the TNFR1 membrane-proximal region. By contrast, the possibility that actin is regulated by multiple signaling pathways cannot be excluded. Actin mutation in L929 cells did not make a global change in TNF signaling as TNF-induced activation of NF-κB and p38 was normal in the mutant line (Fig. 3B).

It has been known for a long time that necrosis is associated with several pathophysiological processes. However, the molecular events that transpire during cellular necrosis remain obscure. An assumption that has limited the study in this field is that necrotic cell death is merely the chaotic breakdown of a cell under intolerable conditions, involving execution mechanisms almost as diverse as the triggers initiating cell death. More and more experimental evidence suggests that at least some necrotic cell death has a conserved core execution program. Our data runs starkly counter to the hypothesis that necrosis is a chaotic breakdown because, in our study, a partial lack of actin, which is a major cytoskeleton component, did not make the cells easier to 'breakdown' but rather inhibited this process. By contrast, disruption of actin filament integrity promptly induced apoptosis in some cells (White et al., 2001). Our data suggest that β-actin is involved in a cellular program that leads to mitochondria responses in TNF-treated L929 cells.

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