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Actin Cleavage in Various Tumor Cells is not a Critical Requirement for Executing Apoptosis

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Actin is a major cytoskeletal protein which is involved in many physiological cellular functions such as motility, cell shape, and adhesion. Recently, actin has also been reported to be cleaved by apoptotic proteases (i.e., caspases) and this cleavage is thought to contribute to the apoptotic process. However, conflicting data also exists as to whether actin represents a true caspase substrate during apoptosis induction in vivo (i.e., inside the cells). In this study, we critically examined the actin cleavage patterns during apoptosis of several tumor cell lines derived from three different species (i.e., mouse, rat, and human). Our findings demonstrate that: 1) actin cleavage in vivo is not a common phenomenon since apoptosis caused by multiple inducers in most cell types examined occurs without evidence of actin degradation; and 2) in certain cell types (e.g., U937), spontaneous, actin cleavage is observed which is not prevented by various specific chemical/peptide inhibitors of proteases such as caspases or serine proteases although apoptosis per se is retarded by some of these inhibitors. Our results conclude that actin is not a critical substrate for apoptotic proteases in vivo during apoptosis. (Pathology Oncology Research Vol 4, No 2, 135–145, 1998)

Key words: actin cleavage, apoptosis, caspase

Introduction

Apoptosis is now implicated in suppression of cancer and virus infection, and its inappropriate activation is associated with many other diseases, including stroke, heart attack, Alzheimer's, AIDS, and aging.[1] The execution of apoptosis involves a series of morphological changes and an ordered sequence of biochemical events. Morphological features include cell shrinkage, membrane blebbing, organelle relocation, chromatin condensation, and cell fragmentation into apoptotic bodies. The activation of chromatin degradation (i.e. into distinct ladders) is perhaps one of the most characteristic biochemical features of apoptosis, although it occurs as a mid-to-late event in the execution of the death program. However, even though many of the biochemical events that occur during apoptosis have been identified, mechanisms by which these events are triggered, regulated and coordinated are still poorly understood. Multiple lines of evidence indicate that proteases, particularly caspases (cysteinyl-aspartate-specific-proteinases) play an essential role in apoptosis.[2,3] Roughly, three classes of caspases have been identified according to their respective functions. Pro-inflammatory caspases, i.e. caspase-1, -4, and -5, are primarily involved in activating cytokines such as interleukin-1 and may not participate in apoptosis. Initiating caspases, i.e. caspase-2, -8, -9, and -10, are localized at the proximal end of the apoptotic signal transduction and are mostly responsible for cleaving and activating downstream caspases. Lastly, executioner caspases, i.e. caspase-3, -6, and -7, are localized at the distal end of the apoptotic signal transduction. These caspases are probably the most important proteolytic enzymes that confer on the dying cells with the typical apoptotic morphologies.

It is generally accepted that caspases participate in apoptotic programs by inducing proteolytic degradation of

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Table 1. **Proteolytic substrates during apoptotic induction.**

Proteins	*Inducer(s)*	*Caspase(s)*	*Cleavage Site(s)*	*Inhibited by*	*References*
Cytoplasmic					
Regulatory proteins					
p21 WAF 1	TNF-α	N/A	N/A	YVAD	4
PKC-δ	Radiation, TNF-α	ICE	$DMQD^{330}N$	YVAD, Bcl-2, Bcl-X_L	5–6
PKC-θ	ara C, cisplatin, VPl6	caspase-3	$DEVD^{354}K$	Bcl-X_L, Crm A, p35	7
Bcl-2	overexpression	caspase-3	$YEWD^{31}/AGD^{34}/A$	zVAD	8
Bcl-X_L	viral infection	caspase-3; caspase-1	$HLAD^{61}$	DEVD,YVAD, Iodoacetamide	69
$cPLA_2$ (Phospholipase A_2)	TNF-α	caspase-3	$DELDS^{522}$	Crm A, DEVD	9
mdm2	starvation, PI-3 K inhibitors	caspase-3	$DVPD^{361}$	DEVD	10–11
D4–GDI (GDP-dissociation inhibitor)	Anti-Fas, staurosporine	caspase-3; caspase-1	$DELD^{19}S$	DEVD, YVAD	12
PRK2 (PKC-related kinase-2)	Anti-Fas, staurosporine	caspase-3	Asp^{117} and Asp^{700}	DEVD	13
Rabaptin-5	in vitro reconstitution	caspase-3	N/A	DEVD	14
SREBP-1/2 (sterol regulatory element binding protein)	Anti-fas, staurosporine, VPl6	caspase-3, caspase-7	SEPD/SP, DEPD/SP	DEVD	15–17
PAK2 (p21 activated kinase 2)	Anti-Fas, TNF-α, ceramide	caspase-3 (?), caspase-1 (?)	Asp^{212}	DEVD, YVAD	18
Pro-IL-1β	Anti-Fas	caspase-1	N/A	YVAD	19
Interferon-γ inducing factor	N/A	caspase-1 caspase-3	Asp^{35} Asn Asp^{69} Ile	YVAD, zVAD	20
Huntingtin	Camptothecin, Apoptotic extract	caspase-3; caspase-1	$DSLDS^{513}$	DEVD	21,72
Atrophin-1	Apoptotic extract	caspase-1/3/7/8	Multiple sites	DEVD	72
Ataxin-3	Apoptotic extract	caspase-1; caspase-3	Multiple sites	N/A	72
Androgen receptor	Apoptotic extract	caspase-1/3/7/8	Multiple sites	DEVD	72
MEKK1 (MEK kinase 1)	Radiation, Anti-Fas	caspase-3	$^{871}DTVDB^{874}$	DEVD	22,70
Mstl (Serine-threonine kinase)	Anti-Fas	caspase-3	$DEMD^{326}S$	zVAD, DEVD	73
Presenilin (PS2)	VP16, staurosporine	caspase-3	Asp^{326} or Asp^{329}	zVAD, DEVD	23
Structural proteins					
Actin	VP16, cisplatin	caspase-3, caspase-1 (?)	Asp^{11}/Asn^{12} Asp^{244}/Gly^{245}	YVAD, TPCK, 24–26 z-Asp, zVAD, zEVD TLCK, iodoacetamide	
	Starvation	ICE	Asp^{11}/Asn^{12} Asp^{244}/Gly^{215} Glu^{107}/Ala^{108}	YVAD	27
	Spontaneous	calpain	Val^{43}/Met^{44}	ALLN	28,71
	Inactivation of T-Antigen	ICE-like	N/A	YVAD	29

ABP (actin binding proteins)					
Gas2	Starvation, cisplatin, radiation	ICE-like	RVD[279]GK	N/A	30
α-actinin	Anti-CD3	calpain	N/A	calpectin, CBZ-LLY, CBZ-LVG	31
gesolin	Anti-Fas, TNF-α, cycloheximide	caspase-3	Asp352/Gly353	zVAD	32
β-catenin	Serum withdrawal	caspase-3	Asp[114]	DEVD	72
FAK	Ant-Fas, TRAIL	caspase-6	VSWD[704]S	DEVD, zVAD	33–34
(focal adhesion kinase)		caspase-3/7	DQTD[772]S		
Fodrin	Anti-CD3, Anti-Fas, ceramide, staurosporine, TNF-α, cycloheximide	caspase-1	N/A	VAD, YVAD	35–37
Keratin 18	VP16	caspase-6 caspase-3/7	VEVD[238]/A	N/A	38–39
Nuclear					
Regulatory proteins					
Rb	VP16, ara C, TNF-α, Anti-Fas, staurosporine	ICE-like; caspase-3-like	[883]DEAD[886]	YVAD, iodoacetamide	40–43
PITSLRE kinase (Cdc-2 kinase)	TNF-α	caspase-1; caspase-3	YVPD[393]S	Bcl-2, Crm A	44
NF-κB	Anti-Fas	caspase-3	N/A	DE VD	45
IxB-α	N/A	caspase-3	D[31] SGLDS	DEVD	46
U1-70kDa	Anti-Fas, TNF-α, UVB, starvation	caspase-3; caspase-1(?)	N/A	DEVD, TPCK, TLCK, iodoacetamide	47–48
PARP	Multiple	caspase-1, -4, -6, -7	DEXD	DEVD, zVAD	49–51
DNA-PK	UVB, staurosporine	caspase-3	DEXD	DEVD	52–54
hnRNP CI, C2	ceramide, VP16	caspase-3, 6, 7	N/A	DEVD, TPCK, TLCK	55
DSEB/RF-C140 (DNA replication factor)	Anti-Fas	caspase-3	Asp[706]/Gly[707]	DEVD, iodoacetamide	56
MCM3 (nuclear replication factor)	Anti-Fas, staurosporine camptothecin, TNF-α, VP 16	caspase-3	DEFD	TPCK, DEVD, Zn zVAD	57
ICAD (inhibitor of caspase-activated deoxyribonuclease)	TNF-α, staurosporine, Anti-Fas	caspase-3	DEPD[117] DAVD[224]	N/A	58
DNA topo I, II	Anti-Fas	caspase-3	N/A	zVAD	59
Structural proteins					
Lamins	Multiple	cas ase-6; caspase 1 (?)	EVD[230]NG	YVAD, TLCK	60–66
NuMA (nuclear matrix protein)	TNF-α, staurosporine, hydroxyurea	N/A	N/A	TPCK	59, 67
SAF-A (nuclear scaffold attachment factor A)	staurosporine, Anti-Fas	caspase-3(?)	DENGD[130](?)	zVAD, YVAD,	68
			DELGD[143](?)	DEVD, TPCK	

*N/A, not available.

specific cellular substrates. So far, more than forty different cellular proteins have been shown to be specific targets of the executioner caspases.[3] *(Table 1)* From data in *Table 1*, the following general conclusions can be obtained: 1) In most cases, the proteolytic degradation of the reported substrates was characterized using an in vitro reconstitution system(s) and the precise role of these substrate cleavages in apoptosis has not been fully addressed. 2) These proteolytic substrates include both structural and regulatory components inside either the cytoplasmic or nuclear compartment. Functionally, these proteins encompass signal tranducers (e.g., PKCδ, PRK2, DNA-PK), cell cycle regulators (e.g. $p21^{WAF1}$, Rb, cdc-2 kinase), cytoskeletal proteins (e.g., actin, β-catenin, keratin), nuclear skeletal proteins (e.g., lamin, NuMA), transcription factors (e.g., SREBP, NF-κB), DNA replication factors (e.g., MCM3, DSEB/RF-C140), protein translocation machineries (e.g., Rabaptin-5) as well as apoptosis proteins (e.g. Bcl-2, Bcl-X_L). 3) In majority of the cases, the caspase degradation of the substrates has been shown to contribute to the apoptotic process. 4) Multiple apoptotic inducers have been used in the studies presented in *Table 1*, which can be classified into: a) TNFα/Fas, b) growth factor withdrawal (i.e., serum starvation), c) DNA damaging agents including irradiation, VP16, camptothecin, etc., and d) other chemical inducers (i.e., staurosporine, cycloheximide). 5) With respect to the involvement of various apoptotic executioners, caspase-3 is the predominant player in degrading different substrates. Less frequently, caspase-6 and caspase-7 are also involved. It is noteworthy that caspase-1 (i.e. ICE) has also been shown to cleave a variety of substrates in vitro. However, it has not been determined whether caspase-1 mediated cleavage is truly important for apoptosis induction in vivo since ICE is mostly recognized as an inflammatory caspase.[1-3] 6) Different executioner caspases recognize relatively specific proteolytic sites with a preferential requirement for the P1 site being Asp. Generally speaking, DEXD represents the recognition site for the effector caspases (caspase-3/6/7) and W/LEHD for ICE-like caspases (caspase-1, -7) The inhibitor profiles vary with different executioner caspases. DEVD-CHO is a specific inhibitor of caspase-3 while YVAD-CHO is a relatively specific inhibitor of caspase-1. zVAD-fmk inhibits a wide spectrum of caspases while iodoacetamide preferentially inhibits caspase-1.

In this study we critically analyzed the cleavage of one of the substrates, i.e. actin, during apoptosis induction. Actin has been reported to be a substrate for various caspases during apoptosis induction and the cleavage of actin has been hypothesized to allow for the activation of DNase I leading to the subsequent fragmentation of DNA.[27,71] The 45-kDa actin has been shown to be cleaved at two sites (Asp^{11}-Asn^{12} and Asp^{244}-Gly^{245}) to generate ≈41, 30, 14 kDa fragments by ICE/ICE-like proteases.[27] The 41 and 30 kDa bands but not the 14 kDa were recognized by a monoclonal anti-actin antibody against the residues 23-34.[27] *(Fig 1a,b)*. It also has been reported that actin is cleaved at Glu^{107}-Ala^{108} giving rise to a minor 30-kDa band which is recognized by a polyclonal antibody against the residues 364–375.[27] *(Figure 1c)*. Whether this cleavage results from caspase effect is not known.[7] CPP-32/apopain or caspase-3 has also been reported to cleave actin at Asp^{244}-Gly^{245} to generate ≈30 and 15 kDa fragments.[26] *(Figure 1a)*. On the other hand, an actin fragment of ≈38 kDa results from the cleavage at Val^{43}-Met^{44} by calpain-like proteases.[28] *(Figure 1d)* However, these different patterns of actin cleavage have not been consistently observed in other cell systems and, in fact, conflicting data has been reported in the literature.[74] In addition, both in vitro and in vivo experiments have not conclusively shown whether actin is a true substrate of apoptotic proteases and the role of actin cleavage in apoptosis remains unknown. Therefore we attempt, in the present study, to explore the potential relationship of actin degradation and apoptosis in multiple cell types with different inducers.

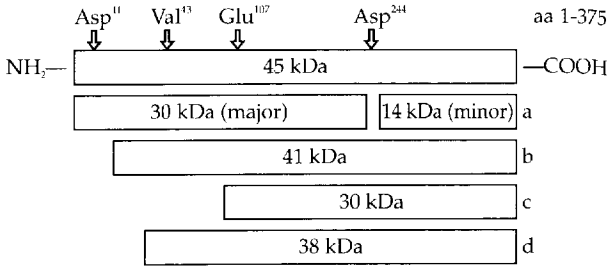

Figure 1. *Representation of actin fragments generated by protease cleavage. Adapted from ref. 26–28. Refer to text for details.*

Our previous work has shown that the actin microfilament contents can be increased in certain tumor cells in response to lipid signaling molecules.[75] We hypothesized that the increased actin filaments may provide a growth/survival advantage to the cells since it has been previously reported that actin is degraded during apoptotic death.[24-26] The present study was thus designed to investigate the potential role of actin cleavage during apoptosis induction by a wide spectrum of triggers. The results demonstrate that actin cleavage in vivo (i.e., inside the cells) is not a prevalent phenomenon. This suggests that actin degradation may not play a critical role in apoptosis.

Materials and Methods

Cell culture, chemicals and reagents

Murine B16a melanoma and rat W256 carcinosarcoma cell lines were maintained with MEM containing 5% fetal bovine serum (FBS) as described.[75-78] Human monocytic

lymphoma U937 cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and antibiotics. Myeloid HL-60 leukemia cell lines were maintained in RPMI-1640 medium containing 10% FBS, 2 mM L-glutamine and antibiotics. Human ovarian carcinoma cells (OVCAR-3) were maintained in RPMI-1640 medium containing 20% FBS and 10 μg/ml insulin and antibiotics. All human cell lines were cultured at 37°C under 5% CO_2. Apoptosis-inducing agents including etoposide (VP16), vinblastine, cisplatin, and staurosporine were obtained from Sigma. Protease inhibitors TPCK (tosyl-L-phenylalanine chloromethyl ketone), TLCK (tosyl-L-lysine chloromethyl ketone), ALLN (acetyl-leucyl-leucylnormethional) and iodoacetamide were purchased from Calbiochem. NDGA (nordihydroguaiaretic acid) and BHPP (N-benzyl-N-hydroxy-5-phenylpentanamide) were used to induce cell death as previously described.[76-78] Peptide inhibitors zVAD-fmk and control peptide Phe-Ala-fmk were purchased from Enzyme Systems, Inc. (Dublin, CA). Cell permeable DEVD-CHO and YVAD-CHO peptides were obtained from Biomol. Most of these chemicals were prepared in ethanol or DMSO. Therefore, in all controls throughout the study, either ethanol or DMSO was utilized, with its final concentration in culture media always being less than 0.1%. In all cases, the pretreatment and treatment was performed in normal culture media (i.e, MEM or RPMI) with appropriate amount of serum, unless otherwise indicated. An anti-actin monoclonal antibody recognizing an epitope in the amino terminus of actin was bought from ICN. An anti-actin polyclonal antibody against the C-terminal 11 amino acids (364–375) of actin was obtained from Boehringer Mannheim. The secondary antibody was either goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Biorad).

Apoptosis induction, quantification of cell survival and apoptosis, DNA fragmentation, and apoptosis assays by light microscopy

Cells were cultured in T-25 flasks and induced to undergo apoptosis by exposure to staurosporine (1-2 μM), VP16 (50–100 μM), BHPP (25 μM), NDGA (50 μM), vinblastine (10–100 μM), cisplatin (250 μM), or growth factor deprivation (i.e., serum starvation). Each condition was characterized by light microscopy over an extended time frame (i.e., up to 48 hr) until morphological signs of apoptosis (i.e., cell shrinkage, membrane blebbing, and appearance of apoptotic bodies) were apparent. Cell survival and quantification of apoptosis were determined using a CellTiter MTS cell proliferation kit (Promega) or DAPI staining as described.[77,79] DNA ladder formation assays were performed as detailed before.[76-79]

Subcellular fractionation and Western blotting

The main protocol was detailed before.[69] Briefly, B16a tumor cells were cultured to approximately 90% confluency in T-175 tissue culture flasks, washed with serum-free MEM and incubated at 37°C for 15 min with serum-free MEM containing 0.1 μM 12(S)-HETE, 0.1 μM PMA (phorbol 12-myristate-13-acetate), or an equal amount of ethanol was used as the control. An additional portion of B16a melanoma cells were subjected to 15 min of 90 cGy gamma irradiation. Samples were processed for fractionation and Western blotting as previously described in detail.[75]

Effects of chemical/peptide inhibitors of proteases on actin cleavage and apoptosis

To investigate the involvement of various proteases in actin degradation and apoptosis, U937 cells were pretreated for 1 hr with the following classes of inhibitors: 1) serine protease inhibitors TLCK (0.1 mg/ml) and TPCK (0.25 mg/ml); 2) cysteine protease inhibitor, iodoacetamide (0.25 mg/ml); 3) calpain inhibitor, ALLN (100 μM); and 4) peptide inhibitors of caspases, DEVD-CHO (caspase-3 inhibitor, 5–50 μM), zVAD-fmk (a more general caspase inhibitor, 5–50 μM), YVAD-CHO (caspase-1 inhibitor, 5-50 μM), and Phe-Ala-fmk (a control peptide, 5–50 μM). Following the pretreatment, VP16 was administered to cell cultures to the final concentration of 20 μM and the treatment lasted 3.5 hr. At the end of treatment, apoptosis was quantitated based on the typical morphology of apoptotic cells. Cells were harvested for both DNA fragmentation assays and actin cleavage assays by Western blotting as described above.

Results

Effects of a variety of agents on the actin microfilament content in B16a melanoma cells

We have investigated the independent effects of a lipoxygenase metabolite of arachidonic acid, 12(S)-HETE, and irradiation on the actin microfilament contents in B16a melanoma cells since both agents have been shown to be involved in a wide variety of cellular activities (i.e. adhesion, spreading, motility) which promote metastasis to occur in tumor cells. Western blotting of the cytosolic and cytoskeletal fractions[75] revealed a single band corresponding to actin (~45 kDa) as shown in *Figure 2*. Several independent experiments revealed a significant increase in both the 12(S)-HETE-treated and irradiated cytoskeletal fractions whereas no substantial alterations were observed in corresponding cytosolic fractions *(Figure 2)*. Similarly, B16a cells treated with PMA, a potent

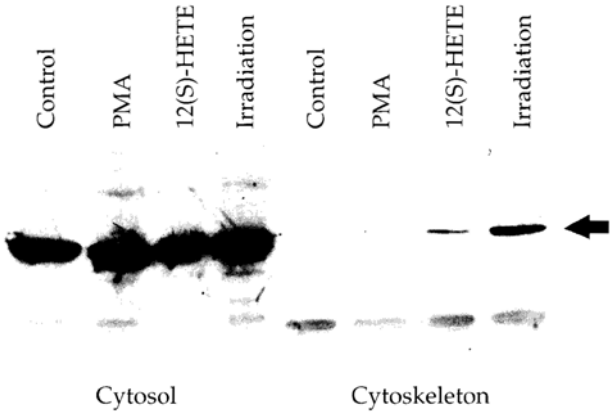

Figure 2. *12(S)-HETE induces an increase in the actin microfilament contents in B16a melanoma cells. Equal number of B16a cells were preteated in MEM-1%FBS with ethanol control, PMA (0.1 μM x 15 min), 12(S)-HETE (0.1 μM x 15 min) or gamma irradiation (15 min, 90cGy). Cells were harvested and fractionated as described in ref. 69. Western blotting was performed with the monoclonal antibody to actin.*

tumor promoter and PKC activator,[80] demonstrated an increase in the F-actin content in the majority of experiments performed even though a prominent increase was not observed in this study *(Figure 2)*. Since these observations suggest that microfilament reorganization may promote tumor cell adhesion and spreading, we further explored the possibility that this system might also play a role in modulating cell survival considering that microfilament assembly has been proposed to be involved in the formation of apoptotic bodies.[81] To test this possibility, we examined the sensitivity of B16a cells to several physiological and chemical inducers. B16a cell apoptosis was observed when treated with vinblastine (a microtubule disrupter), staurosporine (a promiscuous protein kinase inhibitor), BHPP (a 12-lipoxygenase inhibitor), VP16 (a topoisomerase II inhibitor), actinomycin D (a RNA synthesis inhibitor), cycloheximide (a protein synthesis inhibitor), methotrexate (DHFR inhibitor), cisplatin (a DNA alkylating agent), hydrogen peroxide (a pro-oxidant), and serum starvation.[82] However, when control and treated B16a cells were employed in Western blotting experiments, no specific actin cleavage fragments were detected.[82]

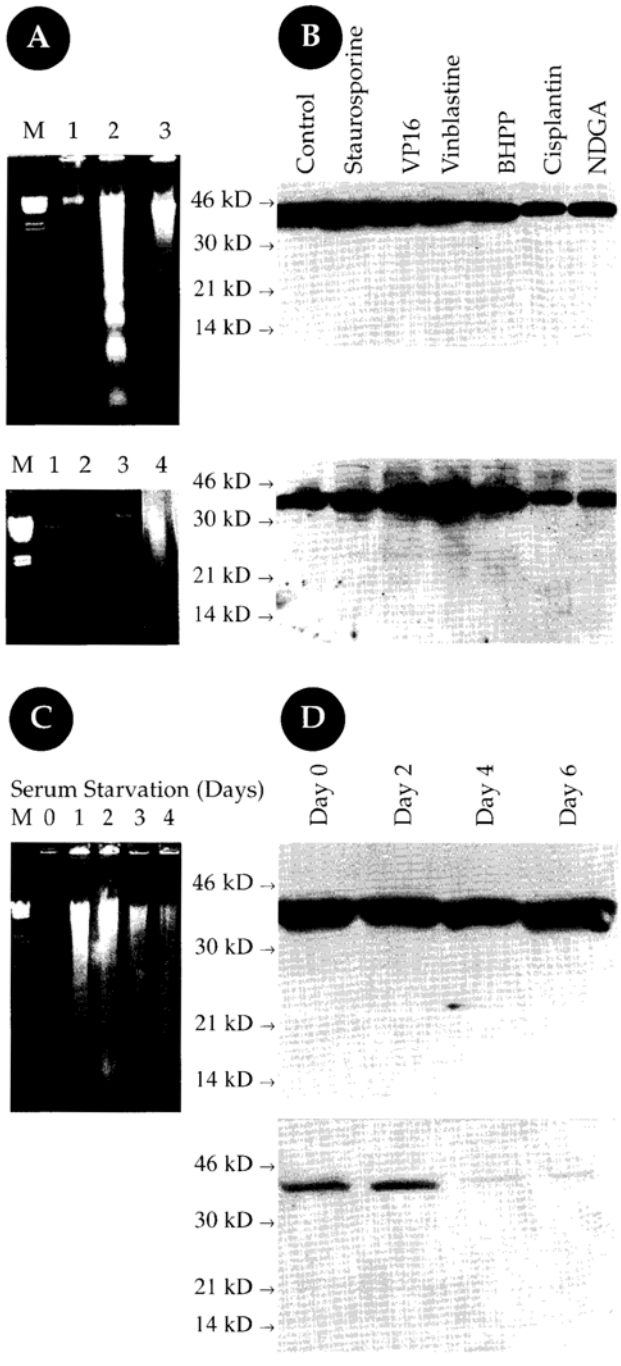

Figure 3. *a-b. Lack of degradation of actin during apoptosis of W256 carcinosarcoma cells induced by multiple agents. A) Equal number of W256 carcinosarcoma cells were treated in MEM-1% FBS with ethanol, (upper and lower panels; lanes 1), staurosporine (1 μM x 8 hr; upper panel, lane 2), BHPP (25 μM x 12 hr; upper panel, lane 3), etoposide or VP16 (50 μM x 24 hr; lower panel, lane 2), vinblastine (10 μM x 24 hr; lower panel, lane 3) and cisplatin (25 0 μM x 20 hr; lower panel, lane 4). Lane M is the molecular weight marker. B) The upper panel shows an immunoblot with the monoclonal antibody against amino-terminal region of actin. The lower panel illustrates the same immunoblot probed with a polyclonal antibody against the C-terminal 11 amino acid of actin. C-D) Lack of actin cleavage activity during apoptosis of W256 carcinosarcoma cells induced by serum withdrawal. C) DNA fragmentation assay. Lane M is the molecular weight marker. Lanes 0–4 represents W256 serum-starved for 0-4 days, respectively. D) Western blotting of actin using the monoclonal (upper panel) and the polyclonal (lower panel) anti-actin antibodies.*

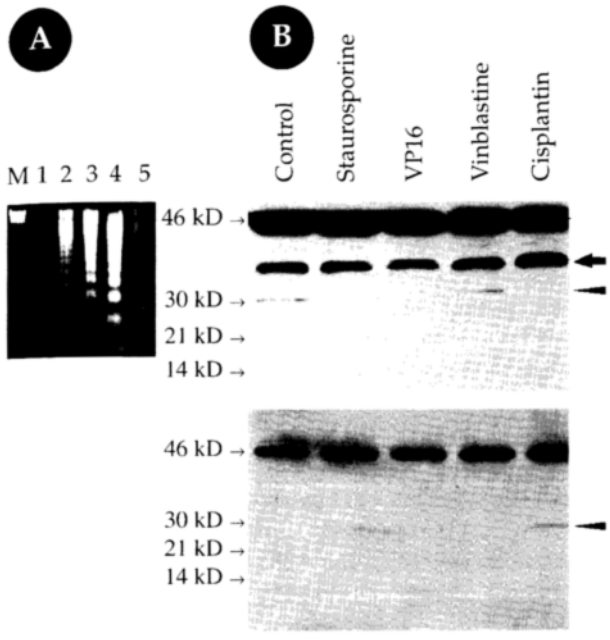

Figure 4. *Actin cleavage in HL-60 leukemia cells during apoptosis induction. a) DNA fragmentation assay. Equal number of HL-60 cells was treated in RPMI-1%FBS with ethanol (lane 1), staurosporine (2 μM x 4 hr; lane 2), VP16 (100 μM x 3 hr; lane 3), vinblastine (100 μM x 4 hr; lane 4) or cisplatin (250 μM x 4 hr; lane 5). b) Western blotting of actin using the monoclonal (upper panel) and polyclonal (lower panel) anti-actin antibodies. The arrow points to the ~38 kDa fragment (upper panel) and the arrowheads to the ~30 kDa bands.*

Lack of actin degradation during the apoptosis of rat W256 carcinosarcoma cells by multiple inducers

We next studied the actin cleavage in W256 carcinosarcoma cells of monocytoid origin since we previously reported that these cells undergo typical apoptotic death in response to many inducers such as NDGA and growth factor withdrawal.[76-78] W256 cells demonstrated typical apoptotic morphology (i.e. membrane blebbing, apoptotic bodies; data not shown) and DNA fragmentation *(Figure 3a)* when treated with commonly used inducers such as staurosporine *(Figure 3a;* upper panel, lane 2), BHPP *(Figure 3a;* upper panel, lane 3), etoposide or VP16 *(Figure 3a;* lower panel, lane 2), vinblastine *(Figure 3a;* lower panel, lane 3) and cisplatin *(Figure 3a;* lower panel, lane 4).

When control and treated W256 cells were employed in Western blotting experiments, both a monoclonal that recognizes the amino-terminus (epitope unknown; *Figure 3b*, upper panel) and a polyclonal that recognizes the C-terminal 11 amino acids *(Figure 3b,* lower panel) of actin failed to detect specific actin cleavage fragments in vivo.

Like most eukaryotic cells that depend on certain trophic factors for their survival, W256 cells underwent typical apoptosis as earlyas 24 hr when deprived of serum growth factors as shown by DNA fragmentation assays *(Figure 3c).* However, we found no evidence for actin degradation in vivo (using both monoclonal and polyclonal actin antibodies) up to six days post-serum deprivation *(Figure 3d).* Interestingly, another rat cell line, PC12, cultured in the absence of serum, a condition that was previously shown to activate actin degradation[27] exhibited random cleavage activity during apoptosis (data not shown).

Serum withdrawal does not cleave actin during human OVCAR-3 ovarian carcinoma cell apoptosis

Next we examined multiple human cell types for actin cleavage during apoptosis. In the absence of serum, OVCAR-3 cells can survive up to four days without any evidence of apoptotic cell death. After four days, OVCAR-3 cells demonstrated typical morphological signs of apoptosis (data not shown) but did not exhibit any actin cleavage activity after seven days post serum removal (data not shown).

Similarly, another human cell line of a different cancer type, prostrate carcinoma (PC-3), also demonstrated typical morphological signs of apoptosis after three days of serum withdrawal but did not exhibit any actin cleavage activity after six days post serum removal (data not shown).

Actin cleavage during human HL-60 leukemia cell apoptosis

In contrast to what we previously observed in other human cell lines (i.e. OVCAR-3, PC3), we did see actin degradation occurring in human HL-60 leukemia cells. HL-60 cells underwent apoptosis in response to staurosporine, etoposide, vinblastine, and cisplatin as revealed by DNA fragmentation *(Figure 4a;* note the DNA fragmentation in cisplatin-treated cells was not very obvious in this experiment). As to our surprise, we detected a ~38 kDa cleaved actin fragment when using the monoclonal antibody against actin *(Figure 4b;* upper panel, arrow). This band was also detected in the non-treated control. With this same antibody, we further observed a ≈30 kDa fragment in both the control and vinblastine-treated but not in staurosporine, VP16, and cisplatin-treated cells *(Figure 4b;* upper panel, arrowhead). In contrast, with the polyclonal anti-actin antibody we detected a fragment of ≈30 kDa in staurosporine-, VP16-, and cisplatin-treated cells but not in control or vinblastine-treated cells *(Figure 4b;* lower panel, arrowhead).

Actin cleavage during human U937 leukemia cell apoptosis

U937 cells underwent apoptosis in response to genotoxic agent VP16 *(Figure 5a;* upper and lower panel, lanes 2). The VP16 induced DNA fragmentation was inhibited or partially inhibited by DEVD (a CPP-32 inhibitor), zVAD (a wide spectrum caspase inhibitor), TLCK and TPCK (serine protease inhibitors) but not by ALLN (calpain inhibitor) or the control peptide, Phe-Ala-fmk *(Figure 5a)*. In pilot experiments, a series of different doses of protease inhibitors were used to treat U937 cells prior to VP16 stimulation. Specifically, TLCK, TPCK, and iodoacetamide at 0.01- 0.5 mg/ml, ALLN at 5–100 μM, and peptide inhibitors (i.e. zVAD, YVAD, DEVD, Phe-Ala-fmk) at 5–50 μM were examined and, subsequently an optimal concentration was used for each inhibitor *(Figure 5)*. Consistent with DNA fragmentation data, the VP16-induced U937 cell apoptosis, as revealed by DAPI staining, was significantly inhibited by pretreatment with DEVD, zVAD, YVAD (caspase-1 specific inhibitor) as well as by TPCK and TLCK but not by ALLN and Phe-Ala-fmk *(Figure 5c)*. When the samples treated as above were employed in the Western blotting with the monoclonal anti-actin antibody, a ≈38 kDa cleaved fragment was detected in the all the samples *(Figure 5b;* upper panel, arrow). Furthermore, a ~30 kDa fragment was detected at various levels under all treatment conditions *(Figure 5b;* upper panel, arrowhead; note that this band became obvious detected in control and VP16-treated samples following longer exposure of the film). Interestingly, the pretreatment of U937 cells with TLCK, and less dramatically with TPCK, increased the intensities of both 38 and 30 kDa bands *(Figure 5*, upper panel). Reprobing the same membrane with the polyclonal anti-actin antibody revealed no specific cleavage products *(Figure 5b.;* lower panel).

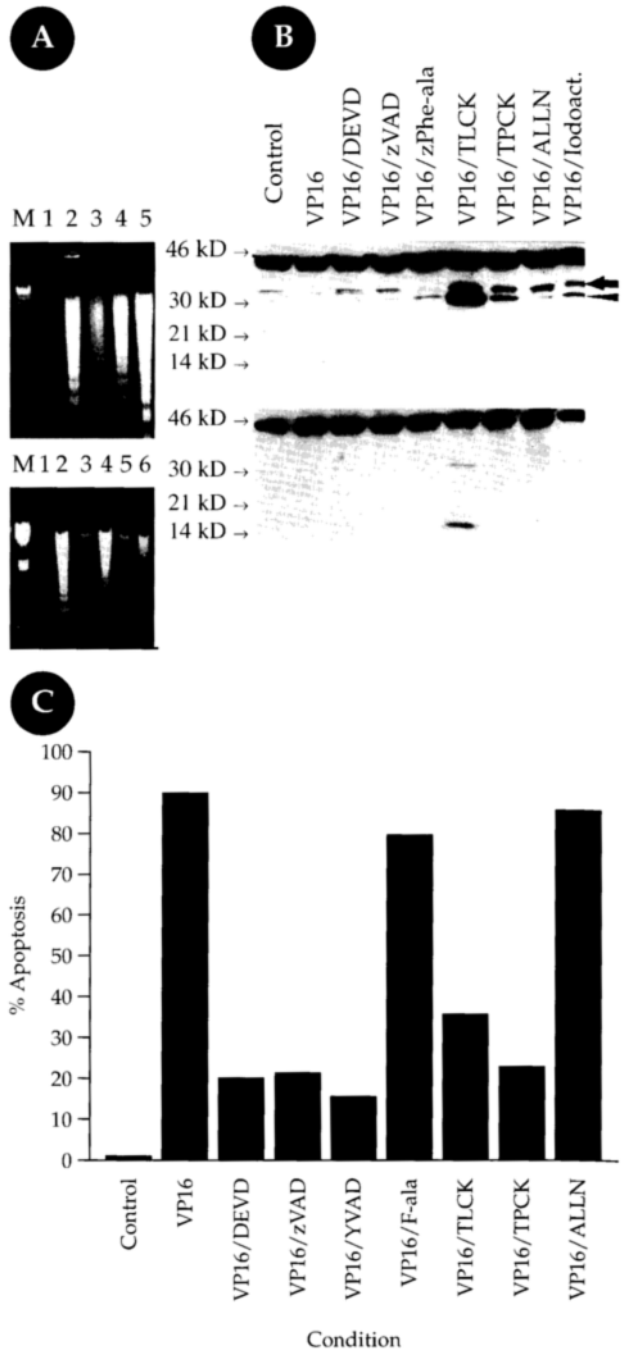

Figure 5. *Actin cleavage during apoptosis in U937 leukemia cells. A) DNA fragmentation of U937 apoptosis. Lane M represents the molecular marker and lane 1 represents DMSO alone (upper and lower panels). U937 cells were pretreated for 1 hr in RMPI-2%FBS with the following inhibitors: DEVD-CHO (50 μM; upper panel, lane 3), zVAD-fmk (25 μM; upper panel, lane 4), Phe-ala-fmk (50 μM; upper panel, lane 5), TLCK (0.1 mg/ml; lower panel, lane 3), ALLN (100 μM; lower panel, lane 4) , TPCK (0.25 mg/ml; lower panel, lane 5), or iodoacetamide (0.25 mg/ml; lower panel, lane 6). Following the pretreatment, VP16 (20 μM) administered to cell cultures for another 3.5 hr. At the end of treatment, apoptosis was quantitated based on the typical morphology of apoptotic cells. Lane 2 (upper and lower panel) represents VP16 treatment (20 μM x 3.5 hr) alone. Note that iodoacetamide induced necrosis of U937 cells. B) Western blotting of actin using the monoclonal (upper panel) and polyclonal (lower panel) anti-actin antibodies. The arrow points to the ~38 kDa fragment and the arrowhead to the ~30 kDa band (upper panel). C) Graphical representation of the number of apoptotic U937 cells after treatment with the above mentioned inhibitors with the addition of YVAD-CHO (25 μM). Quantification of apoptosis was carried out by enumerating apoptotic nuclei following DAPI staining. At least one hundred cells were examined for each condition and assessed for cell shrinkage and nuclear condensation. Apoptotic cells were counted and determined as a percentage of the total number of cells counted.*

Discussion

Recently accumulated experimental data have generated unarguable evidence that caspases, a group of cysteine proteases with aspartate cleavage specificity, play a paramount role in the apoptotic demise of cells. Caspases carry out their functions by specifically and systematically degrading cellular proteins. So far more than 40 caspase substrates have been identified, one of which is actin, an abundant cytoskeletal protein. It has been shown that actin is cleaved by caspase-1/caspase-3 and the actin cleavage has been thought to be important for the apoptotic morphology.[24-28] However, there is also a report that actin normally may not be accessible to caspases and thus is resistant to caspase-mediated degradation.[74] In this study we attempted to address this controversial issue by examining multiple cell types to a wide spectrum of different stimuli.

Specifically, more than 10 different cell lines derived from mouse, rat, and human were analyzed for actin cleavage.[82] The general conclusion is that actin degradation by caspases or other apoptotic proteases is not a common phenomenon in vivo. This is more obvious in solid tumor cells such as B16a, W256, OVCAR-3, and PC3 cells. In leukemic HL-60 cells and in U937 lymphoma cell lines, actin fragments migrating at ~38 kDa and ~30 kDa were indeed observed *(Figure 4–5)*. However, in most cases these bands were also observed in untreated control, suggesting that they may not be apoptosis-related cleavage products. It is well known that blood cells (leukemia/lymphoma cells) have a much higher spontaneous death rate compared to carcinosarcoma cells. Actin has been shown to be cleaved into a 38 kDa fragment by calpain-like protease during spontaneous apoptosis of neutrophils.[28] *(Figure 1)*. Therefore, the 38 kDa band detected in both HL-60 and U937 cells may represent the calpain cleavage product of actin. The nature of the 30 kDa band remains unknown. As shown in *Figure 1*, caspase cleavage at either Glu^{107} or Asp^{244} could generate two 30 kDa fragments of actin which could be detected by polyclonal and monoclonal anti-actin antibody, respectively. In HL-60 cells, the monoclonal anti-actin antibody detected the 30 kDa band fragment in the control and vinblastine-treated cells *(Figure 4b)*. However, this band was not observed in apoptotic HL-60 cells treated by several other inducers. Interestingly, the 30 kDa fragment was detected by the polyclonal antibody only in some inducer-treated cells *(Figure 4)*. Similar to the situation in HL-60 cells, no conclusion can be made regarding the 30 kDa fragment in U937 cells since it was observed more obviously only in inhibitor-pretreated cells but not in control or inducer (i.e., VP16)-treated cells *(Figure 5)*. Taken together, the data suggest that the 30 kDa fragment might represent random cleavage of actin by caspase(s) or some other proteases unrelated to apoptotic activation.

In conclusion, the present study demonstrates that actin cleavage does not represent a prevalent molecular event during apoptosis induction. The data also challenges the notion that actin cleavage is important for apoptosis. Thus, actin, an essential cytoskeletal element, if ever playing a role in programmed cell death, may regulate the apoptotic process in some other aspects such as the formation of apoptotic bodies[81].

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