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Biochemical and Biophysical Research Communications 303 (2003) 37–45
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Caspase-dependent cleavage of tensin induces disruption of actin cytoskeleton during apoptosis☆

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Received 14 February 2003

Abstract

Members of both calpain and caspase protease families can degrade several components of focal adhesions, leading to disassembly of these complexes. In this report, we investigated the disappearance of tensin from cell adhesion sites of chicken embryonic fibroblast cells (CEFs) exposed to etoposide and demonstrated that loss of tensin from cell adhesions during etoposide-induced apoptosis may be due to degradation of tensin by caspase-3. Tensin cleavage by caspase-3 at the sequence DYPD^{1226}G separates the amino-terminal region containing the actin binding domain and the carboxyl-terminal region containing the SH2 domain. The resultant carboxyl-terminal fragment of tensin is unable to bind phosphoinositide 3-kinase (PI3-kinase) transducing cell survival signaling. We also demonstrated that overexpression of the amino-terminal tensin fragment induced disruption of actin cytoskeleton in chicken embryonic fibroblasts. Therefore, caspase-mediated cleavage of tensin contributes to the disruption of actin organization and interrupts ECM-mediated survival signals through phosphatidylinositol 3-kinase.

Keywords: Focal adhesion complex degradation; Tensin degradation during apoptosis; Disruption of actin cytoskeleton

Apoptosis is a fundamental and complex biological process that enables an organism to eliminate unwanted or defective cells through an orderly process of cellular disintegration [1–3]. Apoptosis is characterized by morphological changes including nuclear condensation, cell shrinkage, membrane blebbing, and detachment from extracellular matrix (ECM). The induction of apoptosis involves the proteolytic activation of a cascade of cysteine proteases called caspases. Caspases contribute to cell death by directly inactivating negative regulators of apoptosis and by promoting the disassembly of cellular structures such as focal adhesion complexes [4].

Interaction of cells with ECM is mediated by integrin receptors on the cell surface [5]. Integration of the intracellular signaling and structural responses initiated by ECM-integrin engagement may be mediated through the focal adhesion complex [6]. The focal adhesion complex consists of numerous, tightly associated, structural and signaling proteins, and the cytoplasmic tail of β integrin, thereby providing intracellular signal transduction machinery and also serving as a physical link between the ECM and the cytoskeleton [7]. The structure and functions of focal adhesion complex may influence the signal transduction pathways associated with cytoskeletal rearrangement and play key roles in many biological process.

In addition, the transition from flat to round cell morphology, which is a characteristic feature in cells undergoing apoptosis, is accompanied by cytoskeletal rearrangement and changes in focal adhesion proteins. In epithelial and endothelial cells, disruption of integrin contacts results in the induction of apoptosis [8,9] and the recent studies demonstrated that several focal

☆ *Abbreviations:* CEFs, chicken embryonic fibroblasts; FAK, focal adhesion kinase; ECM, extracellular matrix; ZVAD-fmk, ZVAD-fluoromethylketone; DEVD-cmk, DEVD-chloromethylketone; PENN/MMAC1, phosphatase and tensin homologue on chromosome 10/mutated in multiple advanced cancers.

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doi:10.1016/S0006-291X(03)00280-8

adhesion proteins including focal adhesion kinase (FAK), p130Cas, and HEF1 undergo caspase-mediated cleavage during apoptosis [10,11]. These studies suggest that disassembly of the focal adhesion complex may modulate apoptosis by interrupting survival signals derived from attachment to ECM. In addition to focal adhesion disassembly, apoptosis is accompanied by the dramatic reorganization of the cytoskeleton, which precedes the morphological changes and cellular fragmentation [12]. In particular, several reports indicate that cytoskeletal proteins (such as Gas2, gelsolin, β-catenin, and PAK-2) are proteolytically cleaved by caspases during apoptosis [13–16]. However, little is known about the disruption of the actin cytoskeleton during apoptosis.

Among many focal adhesion proteins, tensin is of particular interest because it plays roles in signal transduction for cell survival as well as in cytoskeleton organization. Tensin is activated and tyrosine-phosphorylated in response to numerous stimuli such as cell adhesion to ECM [17], treatment with platelet-derived growth factor [18], thrombin or angiotensin [19] or transformation by oncogenes such as v-src or bcr/abl [20,21], and influences a variety of cellular functions. Increased tyrosine phosphorylation of tensin can activate both JNK and p38 pathways [22] and its Src homology 2 (SH2) domain is able to interact with phosphoinositide 3-kinase (PI3-kinase) and p130Cas [23,24]. Tensin also shares sequence homology with a tumor suppressor, PTEN/MMAC1 [25]. In addition to its functions as a signal transducer, tensin is a major cytoskeletal protein because it locates in closest proximity to the ends of actin filaments because of its F-actin-binding and capping activities [26,27].

As tensin has been shown to contribute both to the formation of actin cytoskeletal structures and to signal transduction through integrin, we examined the role of tensin in the signal pathway and in the disassembly of actin cytoskeleton during apoptosis. In this study we describe the rapid cleavage of tensin mediated by caspase and/or calpain during etoposide-induced apoptosis. The cleavage of tensin occurs in parallel with disorganization of actin cytoskeleton and caspase-mediated cleavage fragment of tensin containing actin binding domain represents a novel effector of actin cytoskeleton disassembly.

Materials and methods

Materials. Culture media and supplements were purchased from Gibco-BRL (Grand Island, NY). Etoposide was from Sigma Chemical (St. Louis, MO) and enhanced chemiluminescence (ECL) reagent was from Amersham (Buckinghamshire, England). Monoclonal antibody (mAb) against tensin (tensin mAb) and FAK were from Transduction Laboratories (Lexington, KY). Anti-α-tubulin mAb and anti-talin antibody were from Sigma. Chicken tensin cDNA was generously provided by Dr. L.B. Chen (Dana-Faber Cancer Institute, Boston, MA). Horseradish peroxidase (HRP)-labeled anti-mouse and rabbit immunoglobulin and FITC- and TRITC-conjugated goat anti-mouse and anti-rabbit IgG or IgM were from Jackson ImmunoResearch Lab (West Grove, PA). Quick Change Site-Directed Mutagenesis System was from Stratagene (La Jolla, CA). TNT-Coupled Reticulocyte Lysate System was from Promega (Madison, WI). The tetrapeptide caspase inhibitors, ZVAD-fmk and DEVD-cmk, were from Bachem (Torrance, CA).

Cell culture and immunofluorescence. CEFs (chicken embryonic fibroblasts) were isolated from chicken embryos (embryonic day 10) and cultured at 37 °C, 5% CO_2 in DMEM containing 5% fetal bovine serum (FBS), 1% chicken serum, 100 U/ml penicillin and streptomycin, and 2 μM glutamine. CEFs from the third to the eighth passage were used for experiments. For immunofluorescence assay, cells grown on 0.1% gelatin-coated coverslips were fixed with 1.5% paraformaldehyde for 10 min and washed in phosphate-buffered saline (PBS). Cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min and then incubated with the primary Abs and FITC- or TRITC-conjugated secondary Abs. The coverslips were mounted with 90% glycerol and 0.1% *o*-phenylenediamine in PBS. Immunofluorescence was analyzed using a Leica DMRBE microscope equipped with a 100× or 63× objective lens and filters for epifluorescence.

Immunoprecipitation and phosphoinositide kinase assays. PI3-kinase activity was measured either by immunoprecipitation with anti-phosphotyrosine antibody or by anti-tensin polyclonal antibody. The immunoprecipitation was done as follows. After washing twice with ice-cold PBS, cells were solubilized by incubation for 30 min in 1 ml lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium orthovanadate, 100 mM NaF, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 0.75 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 mM PMSF) on the ice. The cell lysates were then cleared by centrifugation at 6000*g* for 10 min at 4 °C. The appropriate antibody was added and incubated at 4 °C on a rocker platform for 2 h. Protein A–Sepharose (50% suspension in phosphate-buffered saline) was added and incubated for 1 h. All complexes were collected by centrifugation and washed three times with 1% Nonidet P-40, 1 mM EDTA, and 100 μM vanadate in phosphate-buffered saline; three times with 0.5 M LiCl and 100 μM vanadate in 100 mM Tris (pH 7.25); and twice with 100 mM NaCl and 1 mM EDTA in 10 mM Tris (pH 7.25). Immunoprecipitates were resuspended in 50 μl kinase assay buffer containing 20 μM Tris (pH 7.6), 75 μM NaCl, 10 μM $MgCl_2$, 200 mg ml^{-1} phosphatidylinositol (sonicated in 20 μM Tris, pH 7.5 as 10 mg ml^{-1} phosphatidylinositol), 1 μM EGTA, 20 μM ATP, and 10 μCi [γ-^{32}P]ATP and incubated for 30 min at room temperature. The organic phase was collected and analyzed by Thin Layer Chromatography (TLC) on Silica Gel 60 plates precoated with potassium oxalate in $CHCl_3$–CH_3OH–H_2O–NH_4OH (60:47:11.3:2, v/v) solvent system.

Electrophoresis and immunoblot analysis. For immunoblot analysis, cells treated as described above were lysed in a lysis buffer [1% SDS, 1 mM sodium orthovanadate, 10 mM Tris (pH 7.4), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 1.5 μM pepstatin, and 10 μg/ml aprotinin], then collected, boiled, and centrifuged for 5 min to remove insoluble material. Protein concentrations in the resultant lysate were measured using the BCA method (Pierce, Rockford, IL). The proteins present in aliquots of lysate were separated by 10–12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked for 1 h at room temperature in buffer containing 5% nonfat dried milk in 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20 (TBST). The membranes were incubated first with primary antibody and then incubated in TBST containing an HRP-conjugated anti-mouse or anti-rabbit immunoglobulin (Jackson ImmunoResearch Lab). Bands were detected using ECL according to the manufacturer's protocol. In some cases, blots were stripped by heating them to 65 °C for 30 min in stripping buffer (100 mM of

2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 6.7) and reprobed.

In vitro assay of cleavage by caspases and calpain. Chicken tensin cDNA was used as templates for in vitro labeling with [^{35}S]methionine (1175 Ci/mmol; NEN, Boston, MA) using a coupled transcription and translation reticulolysate system (Promega, Madison, WI). Recombinant caspases were prepared from bacterial cells (*Escherichia coli*, BL21) according to Kook et al. [11].

To assess caspase-catalyzed cleavage, in vitro-translated tensin was incubated for 90 min at 30 °C with 20 μg bacterial cell lysate containing the respective recombinant caspase. To inhibit the caspase-3 activity, zDEVD-cmk (100 μM) or ZVAD-fmk (100 μM) was applied 1 h prior to the addition of labeled tensin.

To assess calpain-catalyzed cleavage, [^{35}S]methionine-labeled tensin was incubated for the indicated time periods at 30 °C with purified porcine m-calpain (calpain II, Sigma) in PBS. The reaction was initiated by addition of $CaCl_2$ to a final concentration of 1 mM. ALLN (100 μM) was added 30 min prior to the reaction to inhibit the calpain activity. Samples were then subjected to SDS–PAGE and autoradiography.

Site-directed mutagenesis and DNA transfection. Tensin mutants were generated from pcDNA3.0-Tensin [28] using the Quik Change Site-Directed Mutagenesis Kit. Asp residues at positions 474 (VSSD^{474}), 590 (SSLD^{590}), 1226 (DYPD^{1226}), and 1369 (SSPD^{1369}) of chicken tensin were replaced with Glu. Two sets of primers—5′-TGTCG GTGAG CAGCG AGTCG GGCAA CTCCA C-3′ and 5′-GTGGA GTTGC CCGCT TCGCT GCTCA CCGAC A-3′ for the D^{474} mutation, 5′-GCTCT CGTCC TTGGA AGGCA CCACC ACTGC C-3′ and 5′-GGCAG TGGTG GTGCC TTCCA AGGAC GAGAG C-3′ for the D^{590} mutation, 5′-GCCCT GACTA CCCTG AGGGC CGAGG CGGC-3′ and 5′-GCCGC CTCGG CCCTC AGGGT AGTCA GGGC-3′ for the D^{1226} mutation and 5′-ACTCC TCCTC CCCGG AGTCC GCCGC CTACC G-3′ and 5′-CGGTA GGCGG CGGAG TCCGG GGAGG AGGAG T-3′ for the D^{1369} mutation—were used for construction of the mutants. The sequence of the mutants was then confirmed by DNA sequence analysis.

The 5′ fragment of Tensin and full-length wild type Tensin were cloned. Wild type tensin was cloned from pRcCMV-SHL-0 (Courtesy of Dr. Lan Bo Chen, Dana-Farber Cancer Institute, Boston, MA) to pEGFP-C1 vector. The 5′ fragment of tensin was PCR-amplified from pRcCMV-SHL-0 using the forward and reverse primers, 5′-CGCGG ATCCATGGATTTCGGAAGC-3′ and 5′-CGCGGATCCGTCAGG GTAGTCAGG-3′, respectively. The PCR was carried out using pfu DNA polymerase (Stratagene, La Jolla, CA), digested with *Bam*HI, and cloned into pEGFP-C1 (Eastman Kodak, New Haven, CT) to generate pEGFP-C1-5′ Tensin. CEF cells grown in DMEM with 5% FBS, 1% CS, and 1% L-glutamate were transfected with the constructed DNAs using Lipofectamine reagent (Invitrogen, NV Leek, The Netherlands).

Results

Loss of tensin from cell adhesions is concomitant with disruption of actin cytoskeleton during apoptosis

A growing number of intracellular proteins have been shown to associate with focal adhesions. Tensin is of particular interest because it has the ability to interact with actin filaments at multiple sites. To analyze the dynamics of actin cytoskeleton organization and cell adhesions during apoptosis, we performed double immunofluorescence staining to compare tensin distribution in relation to actin cytoskeleton in apoptotic chicken embryonic fibroblasts (CEFs). Intense staining for tensin was observed at cell adhesion contacts in nonapoptotic CEFs in which actin filaments were well developed. (Fig. 1A). At an early apoptotic stage, when a retraction response was observed, tensin staining was found at the cell periphery during the process of cell detachment from the substratum. In control cells, actin stress fibers form well arranged straight lines in the cytoplasm and in peripheral regions, whereas most of the cytoplasmic stress fibers had disappeared in etoposide-treated cells, and only discontinuous, truncated fibers were seen in the cell periphery and then became diffusely cytoplasmic. After 24 h of incubation with etoposide, disruption of the actin organization became clearly detectable as shown by phalloidin staining. Moreover, the altered cellular localization of tensin during apoptosis accompanied the disruption of the actin cytoskeleton. Therefore, disruption of the actin cytoskeleton seems to be concomitant with loss of tensin in cell adhesions.

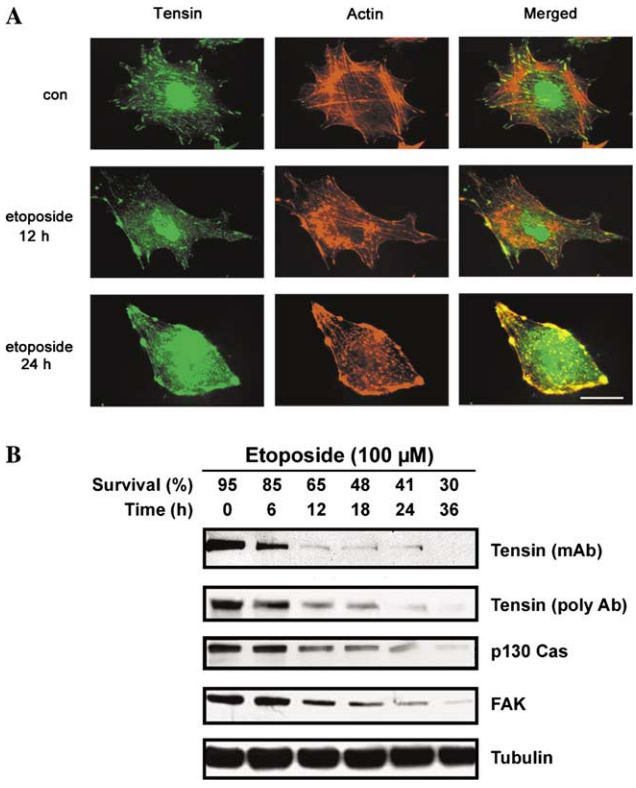

Fig. 1. Loss of tensin from focal adhesion sites during etoposide-induced apoptosis of CEFs. (A) Apoptosis of CEFs was induced by treatment with 100 μM etoposide for 24 h. Tensin was then stained with a polyclonal anti-tensin antibody followed by FITC-conjugated goat anti-rabbit secondary antibody. Actin was detected with TRITC-phalloidin. Images are shown as both separate and merged images. Bar, 10 μm. (B) CEFs treated with 100 μM etoposide for the indicated times were analyzed by Western blotting of cell lysates. The blot was probed with a mAb raised against a N-terminus of tensin, a polyclonal antibody raised against a C-terminal fragment of tensin, and antibodies directed against FAK and tubulin. Cell viability was assessed by trypan blue exclusion.

When CEFs were incubated with 100 μM etoposide, cell survival was markedly decreased after a few hours and apoptotic cells could be easily observed floating in the medium. In order to study tensin cleavage during etoposide-induced apoptosis, both adherent and non-adherent floating cells were combined and Western blot analysis was performed. As shown in Fig. 1B, Western blot analysis with a monoclonal antibody against the NH_2 terminus of tensin demonstrated a significant reduction in the levels of native tensin. Similar results were obtained using polyclonal antibody against the COOH terminus of tensin. Other focal adhesion proteins, FAK and p130Cas, dramatically decreased during etoposide-induced apoptosis (Fig. 1B).

Tensin is cleaved by caspase-3 and/or calpain

To determine whether caspases could be responsible for the proteolytic cleavage of tensin during apoptosis, an in vitro cleavage assay using caspases was performed. Full-length tensin cDNA was translated in vitro and treated with either caspase-1, caspase-3, or caspase-8. As shown in Fig. 2A, treatment with caspase-3 specifically cleaved tensin, producing fragments of M_r ~160K and ~60K, whereas caspase-1 and caspase-8 failed to cleave tensin. Moreover, caspase-3 dependent proteolytic cleavage of tensin appeared to be prevented by both the general caspase inhibitor, ZVAD (100 μM), and a specific caspase-3 inhibitor, DEVD (100 μM) (Fig. 2B).

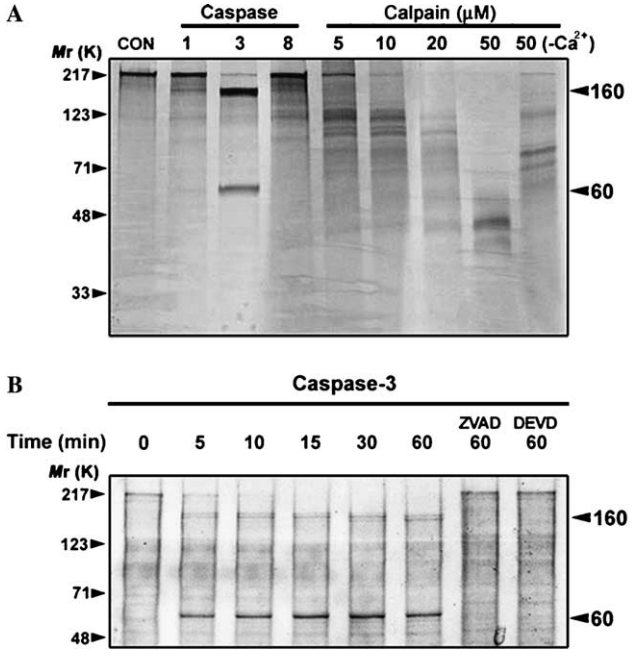

Fig. 2. Tensin is a substrate for caspase-3 and calpain in vitro. (A) ^{35}S-labeled tensin was generated by in vitro translation and incubated with bacterial cell lysate (20 μg) containing overexpressed recombinant caspases (caspase-1, -3, and -8) or purified calpain II at various concentrations. (B) In vitro-translated tensin was incubated with recombinant caspase-3 (20 μg) for the indicated times with or without either ZVAD-fmk (100 μM) or DEVD-cmk fmk (100 μM) to inhibit caspase-3 activity. The arrowheads on the right indicate the cleavage products.

In order to determine whether calpain II is capable of cleaving tensin in vitro, we incubated in vitro translated tensin with purified calpain II. Calpain II induced dose-dependent cleavage of tensin and generated M_r 120K, 85K, and 45K fragments. Tensin cleavage by calpain II was partially calcium dependent (Fig. 2A). These results indicate that tensin may be cleaved by caspase-3 as well as calpain II.

To directly determine whether caspase-3 and/or calpain are responsible for tensin cleavage, CEFs were preincubated with a well characterized pharmacological peptide aldehyde inhibitor of calpain activity, ALLN and/or the broad spectrum caspase inhibitor, ZVAD, for 3 h before the addition of etoposide, and then immunoblot was performed using tensin mAb able to detect the NH_2-terminus. As shown in Fig. 3A, a 160-kDa proteolytic fragment of tensin was easily detected in the etoposide-treated cells. In the presence of ALLN, a calpain inhibitor, the cleavage of native tensin was partially inhibited but was completely inhibited by ZVAD, a caspase inhibitor. When ALLN and ZVAD were used in combination, inhibition of tensin cleavage was substantially greater than that seen for either ALLN or ZVAD alone. Cells without ALLN or ZVAD showed 51% death rate by etoposide treatment. Treatment with ALLN or ZVAD significantly inhibited etoposide-induced apoptosis of CEFs—100 μM ALLN increased the cell survival to 78% and 200 μM ZVAD increased it to 85%. Combination treatment increased cell survival to 90%. Cell shrinkage and loss of cell adhesion were also blocked by addition of ALLN or ZVAD (Fig. 3B). These results indicate that proteolysis of tensin in CEFs is mediated by both caspase and/or calpain II.

Tensin contains a caspase-3 cleavage site

Based on the size of the tensin fragments generated by caspase-3 proteolysis in vitro, we found several putative cleavage sites at $VSSD^{474}S$, $SSLD^{590}G$, $DYPD^{1226}G$, and $SSPD^{1369}S$ and generated point mutations by substituting aspartic acid with glutamic acid (Fig. 4A). The in vitro translated products of these mutants were then incubated with caspase-3. Incubation of the proteins mutated at residues 474, 590, or 1369 with caspase-3 resulted in a cleavage pattern identical with that of wild type tensin. However, the point mutant at D1226E was not cleaved by caspase-3 (Fig. 4B). As shown schematically in Fig. 4A, tensin binds to the barbed ends of actin filaments through three actin-binding domains and also contains a SH2 domain adjacent to the carboxyl terminus. The caspase-3 cleavage site of tensin (at D1226) appeared to be located between

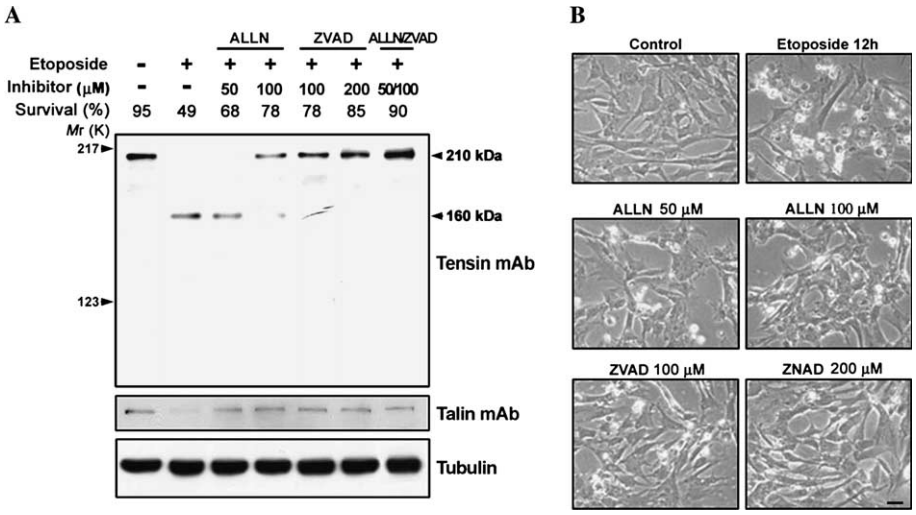

Fig. 3. Inhibition of tensin proteolysis during apoptosis by ZVAD-fmk and ALLN. (A) CEFs were pretreated for 3 h with the indicated concentrations of ZVAD-fmk and/or ALLN and then exposed to 100 μM etoposide for an additional 12 h. The Western blot was probed with an anti-tensin mAb. The same blot was reprobed with an anti-talin antibody and anti-tubulin antibody. (B) Phase contrast micrographs (10× objective lens) of CEFs. Where indicated, cells were pretreated with ZVAD-fmk or ALLN for 3 h prior to treatment with 100 μM etoposide for 12 h. Cell viability was assessed by trypan blue exclusion. Bar, 10 μm.

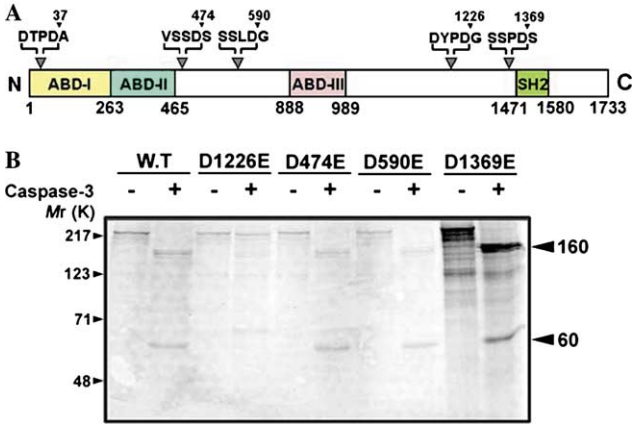

Fig. 4. Tensin cleavage at the sequence DYPD^{1226}G. (A) Schematic representation of the structure of tensin: ABD, actin-binding domain; SH2, src homology 2 domain. (B) Tensin mutants containing point mutations replacing Asp (D1226E, D474E, D590E, and D1369E) with Glu were translated in vitro and incubated with recombinant caspase-3 (20 μg). Cleavage products are shown by the arrowheads on the right.

the three actin-binding sites and the SH2 (Src Homology 2) domain such that the cleavage of tensin at D1226 resulted in the separation of the carboxy terminal SH2 domain from the remainder of the tensin molecule containing three actin-binding sites. It is of interest that cleavage of tensin at D1226 would be expected to separate two functional regions of the protein.

Next, we performed an in vitro cleavage assay of tensin using cell lysates from CEFs treated with etoposide for 6 h as an enzyme source (Fig. 5A). Lysates from apoptotic CEFs, but not from untreated control cells, were able to cleave in vitro translated tensin and generated degradation fragments comparable to those generated by caspase-3. Furthermore, the general caspase inhibitor ZVAD abrogated the ability of the apoptotic CEF cell lysate to cleave tensin whereas the calpain inhibitor ALLN was ineffective. In addition, the previously identified D1226E mutant was not cleaved by lysates derived from apoptotic CEFs. These data indicate that tensin is cleaved primarily by activated caspase-3 during apoptosis of CEFs.

To examine further the role of tensin in the assembly of actin cytoskeleton, cells were transiently transfected with mutant tensin (D1226E) or wild type tensin cDNAs. Immunofluorescence analysis using TRITC-phalloidine in mutant tensin-transfected cells demonstrated that actin cytoskeleton still remained in the cytoplasm and the pheripery of cells after treatment with etoposide for 24 h, whereas actin cytoskeleton in wild type-transfected and untransfected cells was completely lost from the cytoplasm of cells and only detected in the periphery of cells (Fig. 5B).

Functions of caspase-mediated tensin fragment in the apoptotic process

Since tensin has the potential to interact with the actin cytoskeleton and bind with PI3-kinase, we examined the possibility that tensin fragments could modulate disruption of the actin cytoskeleton and cell survival signaling through PI3-kinase from integrins. We first tested the PI3-kinase activity in tensin immunoprecipitates using a polyclonal antibody against the SH2 domain of tensin. As illustrated in Fig. 6A, PI3-kinase activity was present in immunoprecipitates using anti-tensin antibody or anti-phosphotyrosine antibody, and complete inhibition of the activity by LY294002, PI3-

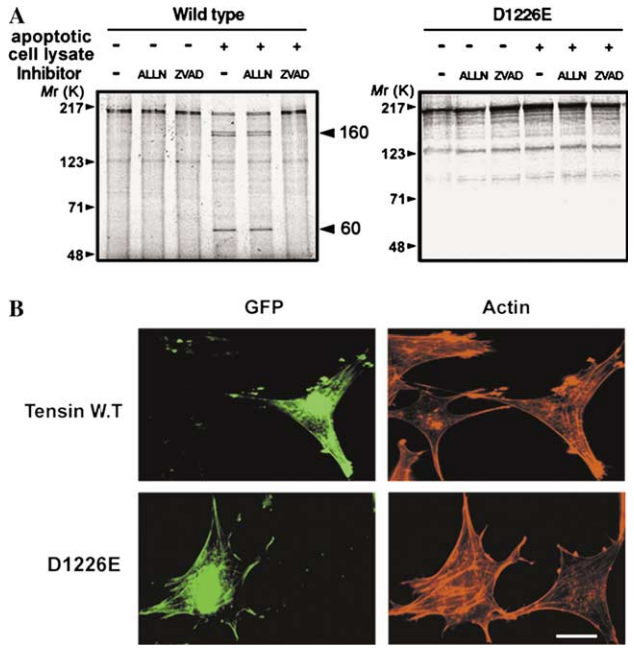

Fig. 5. Cleavage of wild type and D1226E mutant tensin by apoptotic cell lysates. (A) Apoptotic cell lysates (40 μg) were obtained from CEFs treated with etoposide (100 μM) for 6 h and incubated with in vitro-translated wild type tensin or D1226E mutant tensin in the presence of either 100 μM ZVAD-fmk or 100 μM ALLN. (B) Cells were transfected with wild type tensin cDNA or mutant tensin cDNA (D1226E) conjugated with GFP and incubated for 24 h in the presence of 100 μM etoposide. The cells were stained with TRITC-phalloidin to visualize actin cytoskeleton. Bar, 10 μm.

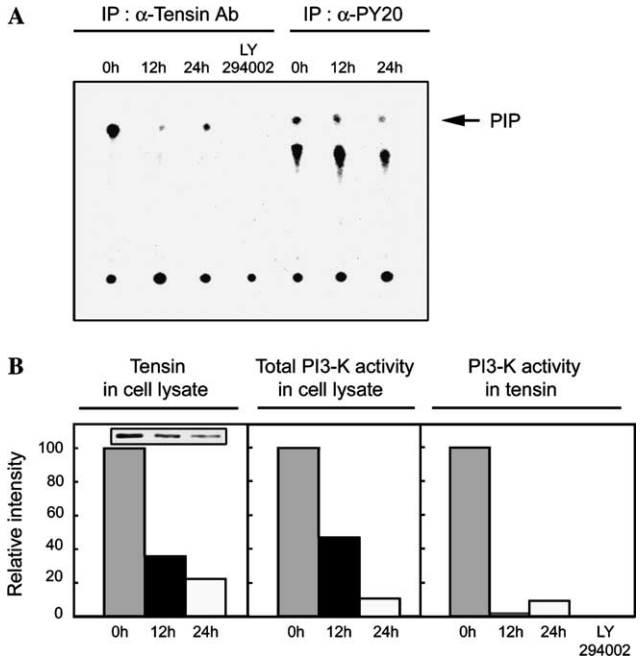

Fig. 6. Change of PI3-kinase activity bound in tensin during apoptosis. (A) CEFs were treated for 0, 12, and 24 h with 100 μM etoposide and cell lysates (100 μg) were immunoprecipitated with a polyclonal anti-tensin Ab raised against the C-terminus of tensin or cell lysates (500 μg) were done with anti-phosphotyrosine antibody (α-PY). Immunoprecipitates were analyzed using a lipid kinase assay. The reaction products from an assay using phosphatidylinositol as substrate were extracted, analyzed by TLC, and exposed to X-ray film. Migration of the ^{32}P-labeled reaction products (phosphatidylinositol phosphate, PIP) is indicated. (B) Change of intact tensin during apoptosis was presented by immunoblot analysis and the band intensity was measured using GS-700 Imaging Densitometer (Bio-Rad). The intensity of spots (PIP) was also measured and the relative intensity was calculated and displayed with bar graphs.

kinase inhibitor, confirmed that phosphatidylinositol phosphate (PIP) was produced by PI3-kinase. PI3-kinase activity present in tensin immunoprecipitates was greatly reduced and less than 10% remained at 12 h after etoposide treatment. To test the possibility that the reduction of PI3-kinase activity bound in tensin is due to the reduction of total PI3-kinase or the reduction of relative amount of tensin during apoptosis, we have measured total PI3-kinase activity and amount of intact tensin present in apoptotic cell lysates. As shown in Fig. 6B, the amount of intact tensin was reduced to 40% at 12 h and 30% at 24 h after etoposide treatment, compared with the control cell without etoposide treatment. PI3-kinase activity present in cell lysates was also reduced to 50% at 12 h after etoposide treatment. The significant reduction of PI3-kinase activity in tensin immunoprecipitates rather than the reduction of total PI3-kinase activity and the reduction of relative amount of tensin during apoptosis suggest that degradation of tensin during apoptosis is likely to inhibit the binding of tensin with PI3-kinase, rendering PI3-kinase incapable of functioning to support cell survival.

Removal of the SH2 domain from tensin by caspase-3 produces the amino terminal fragment containing the actin-binding domain. To determine whether expression of the truncated amino-terminal tensin is sufficient to induce disruption of actin cytoskeleton, we transiently transfected CEFs with a truncated tensin cDNA that encodes an amino terminal of tensin (at position 1–1226). Twenty percentage of cells transfected with the amino terminal fragment of tensin revealed intense disruption of actin cytoskeleton whereas cells transfected with a wild type tensin or GFP showed well-developed actin cytoskeleton (Fig. 7). Percentage of cells transfected with the amino-terminal fragment is low (~20%) and among the transfected cells, 20% cells showed the pattern of disrupted actin cytoskeleton. However, these experiments are not enough to support the fact that expression of the truncated amino-terminal tensin itself is sufficient to induce apoptosis.

Discussion

Cell adhesion to ECM regulates many aspects of normal cell physiology including proliferation, migration, and cell survival [29,30]. Apoptotic cell death is

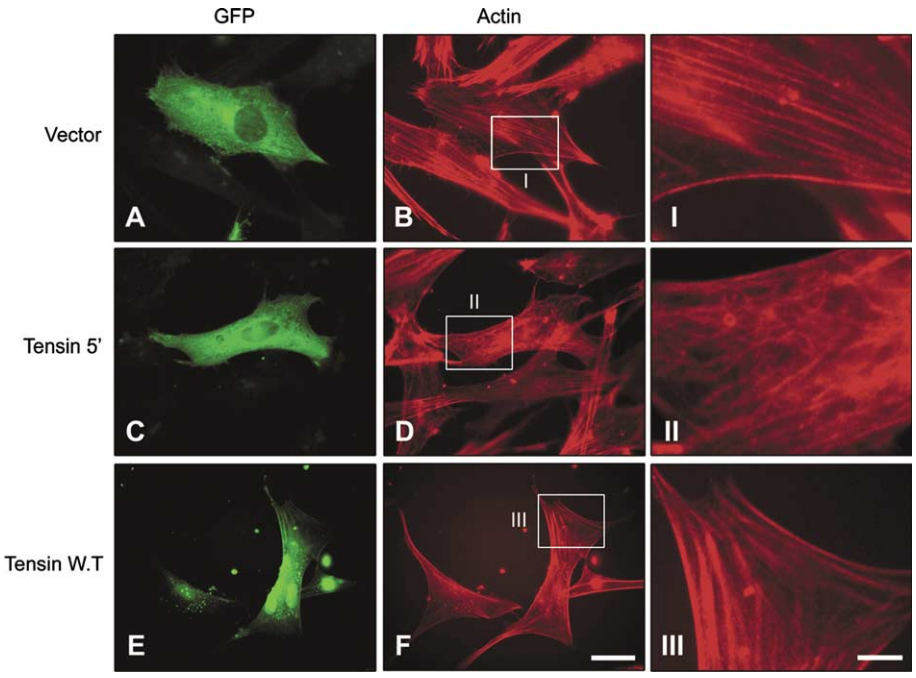

Fig. 7. Disruption of actin cytoskeleton by N-terminal fragment of tensin. Cells were transfected with pEGFP vector only (A and B), pEGFP vector containing cDNAs encoding the NH_2-terminal region (at position 1–1226, C and D) or full length of tensin (E and F). After 24 h, transfected cells were stained with anti-GFP antibody (green) and stained again with TRITC-phalloidin (red) to visualize actin filaments. The left panel (A, C, and E) indicates GFP expression, and the middle panel (B, D, and F) indicates actin staining of the same field. I, II, and III represent a higher magnification of the areas highlighted in B, D, and F. Bars: in H (for A–F), 10 μm; in III (for I–III), 2 μm.

accompanied by dramatic morphologic changes including cell shrinkage and detachment from ECM. Recently, caspase-mediated cleavage of several proteins including gelsolin and ROCK [31,32] has been documented to have direct physiological significance in the morphological changes that are the hallmark of apoptosis.

In the present study, we have demonstrated that tensin is cleaved in etoposide-induced apoptosis of CEFs and that tensin cleavage may be primarily mediated by caspase-3. Considering the potential sites of tensin cleavage, it is reasonable to predict that the $DYPD^{1226}G$ sequence would be preferentially cleaved and generate the 160- and 60-kDa fragments. Using tensin mAb, we were able to detect 160-kDa fragment but not 60-kDa fragment. Interestingly, it is important to note that an additive effect was observed when CEFs were pretreated with both ZVAD and ALLN, implying that both caspase-3 and calpain were involved in tensin cleavage during apoptosis, albeit utilizing different sites. Moreover, the cleavage pattern of in vitro-translated and native tensin is quite different. For example, a 160-kDa fragment appeared at early times (Fig. 3A) which was likely generated by cleavage at $DYPD^{1226}G$ but the fragment quickly disappeared. However, both the 160- and 60-kDa fragments were easily detected in in vitro-cleavage assay using caspase-3 alone. The activity of caspase-3 but not calpain was greatly increased during apoptosis (data not shown) and ZVAD-fmk but not ALLN did prevent cleavage of tensin by apoptotic cell lysates obtained at early time of apoptosis (6 h) in vitro. These results suggest that activity of caspase-3 is more relevant than calpain in the degradation of tensin. However, the previous report by Chen et al. [33] demonstrated the rapid cleavage of in vitro-translated human tensin by calpain II. Although the specific mechanism responsible for the relationship between calpain and caspase-3 during apoptosis is not yet known, calpain activation is likely to be dependent on caspase activation. Similar results have been reported in tumor necrosis factor-induced apoptosis of U937 cells [34] and drug-induced apoptosis of HL-60 cells [35]. Therefore, it is likely that the primary cleavage of tensin occurs at residue D^{1226} by caspase-3 and post-cleavage processing occurs by calpain, thereby producing smaller fragments.

Cleavage of tensin at the sequence $DYPD^{1226}G$ could release both a SH2 domain and an actin binding region that appear to function independently. Tensin is able to interact with certain tyrosine-phosphorylated proteins, such as PI3-kinase and p130cas [23,24]. Signals from PI3-kinase are known to regulate cell survival in response to ECM components. Anoikis resulting from complete disruption of integrin-mediated adhesion involves reduced signaling through PI3-kinase [36]. As shown by our results, interaction of tensin and PI3-kinase was reduced during apoptosis.

Degradation of tensin during apoptosis may thus exert significant downstream effects by interruption of survival signals derived from PI3-kinase activity consequent to interaction with ECM and therefore reduce cell survival.

Several studies have demonstrated that the disassembly and cytoskeletal rearrangement of actin filaments represent significant steps in the process of apoptosis [13,37–39]. Caspase-3-cleaved gelsolin was demonstrated to destabilize the actin network causing cellular retraction, detachment, and apoptosis [31]. Similarly, we demonstrated that tensin disappeared from cell adhesions and that it was concomitant with disruption of the actin cytoskeleton. In addition, overexpression of a tensin fragment containing the actin-binding region in CEFs induced gross alterations of actin organization. Therefore, caspase-mediated tensin degradation is likely to play a proapoptotic effector by release of proteolytic fragments which exert a cytoskeletal disassembling function. Confirmation of this, however, will require further study.

Tensin is phosphorylated at serine, threonine, and tyrosine residues. The tyrosine phosphorylation of tensin occurs when cells are adherent to ECM [17] as well as following transformation of cells with oncogenes such as v-src, bcr/abl [20,21]. According to previous reports [24,26], dimerization and intramolecular folding of tensin are mediated by phosphorylation at tyrosine 393 and subsequent SH2 domain binding may lead to important conformational and biochemical changes in the function of tensin. This conformational change or phosphorylation itself could also directly affect the actin binding domain and thus be important for interactions between tensin and actin. It is therefore of interest that the tensin cleavage site identified in this study is located at $DYPD^{1226}E$ since cleavage at that site would be predicted to separate the actin binding region containing tyrosine 393 and the SH2 domain. As a result of this separation, tensin may not undergo dimerization or intramolecular folding. Confirmation of this scenario, however, will require further study.

In summary, we have demonstrated tensin proteolysis by caspase-3 during etoposide-induced apoptosis and its proteolysis results in the separation of tensin into two fragments such as the actin binding region containing tyrosine 393 and the SH2 domain, and consequently contributes to the morphological changes of apoptotic cells and interrupts ECM-mediated survival signaling through PI3-kinase.

Acknowledgments

Chicken tensin cDNA was generously provided by Dr. L.B. Chen (Dana-Faber Cancer Institute, Boston, MA). This study was supported in part by grants from NRL (National Research Laboratory Program). Dr. S. Kook was supported by the post-doctoral fellowship program of KOSEF, and Do Hoon Kim and Wook Kim were supported by Brain Korea 21 Program.

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