An Autocrine/Paracrine Loop Linking Keratin 14 Aggregates to Tumor Necrosis Factor α-mediated Cytotoxicity in a Keratinocyte Model of Epidermolysis Bullosa Simplex*

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Epidermolysis bullosa simplex (EBS) is a blistering cutaneous disease featuring protein aggregates. Here we investigate the molecular mechanisms linking protein aggregates to cell death in a cellular model of EBS in which HaCaT keratinocytes are transfected with plasmids expressing various mutant forms of keratin 14 (K14). In HaCaT cells, mutant K14 was found to form ubiquitinated protein aggregates that suppressed 20 S proteasome function instead of being degraded by 20 S proteasome. Keratinocytes with mutant K14-induced phosphorylation of the stress-activated kinase c-Jun, as well as up-regulation of unfolding protein Bip, indicates induction of endoplasmic reticulum stress. HaCaT cells were susceptible to apoptosis by activation of caspases-3, -8, -9, and -12. Tumor necrosis factor-α (TNFα) in the culture medium was increased in keratinocytes with mutant K14 compared with wild K14, and the addition of neutralizing anti-TNFα antibody to the culture medium rescued keratinocytes from cell death. Thus, TNFα release and the subsequent activation of the TNFα receptor by an autocrine/paracrine pathway links protein aggregates to cell death in this keratinocyte EBS cellular model. Furthermore, mutation in K14 reduced its affinity to TNFα receptor-associated death domain (TRADD), suggesting that the susceptibility of keratinocytes to caspase-8-mediated apoptosis is increased in mutated K14 because of impairment of the cytoprotective mechanism mediated by K14-TRADD interaction.

Protein folding is the process by which a linear polymer of amino acids is converted to the unique three-dimensional structure of a functional protein molecule. The folding process may fail because of genetic mutations or environmental stresses, such as heat stress (1) and oxidative stress (2), resulting in the formation of protein aggregates. Accumulation of protein aggregates induces endoplasmic reticulum (ER) stress and apoptosis, contributing to the etiology of disorders including neurodegenerative diseases (Alzheimer’s disease, Parkinson’s disease, and prion diseases) and diabetes mellitus. However, the mechanism by which protein aggregates induce ER stress and cell death is not known.

The ubiquitin-proteasome system (UPS) is the major nonlysosomal proteolytic pathway that functions to rapidly eliminate proteins having abnormal conformations (3, 4). Degradation of proteins by this pathway involves two successive steps: (i) covalent attachment of multiple ubiquitin molecules to the target protein, and (ii) degradation of the target protein by the 26 S proteolytic complex. The 20 S proteasome, the catalytic core of the 26 S proteolytic complex, is a cylindrical stack of four seven-membered rings; its proteolytic site faces an interior chamber that can be accessed only through pores at either end of the cylinder. The protein aggregates in several neurodegenerative disorders have been found to be ubiquitin-positive (5). Folded proteins cannot enter the chamber of the 20 S proteasome unless they are unfolded. Because ubiquitinated protein aggregates are barely unfolded, they cannot enter the chamber of 20 S proteasome, and thus are resistant to degradation (6).

Malfolded proteins are unfolded in the ER; accumulation of unfolded proteins in the ER induces an adaptive process, the so-called unfolded protein response (7). The unfolded protein response has at least two distinct components. The first consists of a rapid and transient attenuation of new protein synthesis, which lightens the load on the folding apparatus in the ER. Accumulation of unfolded proteins in the ER phosphorylates PERK, which in turn phosphorylates eukaryotic initiation factor-2α to inhibit further protein synthesis. The second component consists of up-regulation of the expression of genes, the products of which promote protein folding in the ER and the degradation of malfolded proteins. Unfolded proteins in the ER lumen phosphorylate Ire1, activating the endonuclease domain of Ire1 and initiating splicing of mRNA encoding a putative transcription factor, leading to enhanced transcription of ER chaperone genes, including Bip and CHOP. IRE1 phosphorylation also triggers phosphorylation of the stress-activated kinase c-Jun, leading to enhanced transcription of ER.

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chaperone genes. ER stress causes apoptotic cell death (8–11). Although the pathways linking ER stress to apoptosis are still controversial, activation of caspase-12 (12) and/or assembly of IRE1/TRAF2/ASK1 are thought to be involved (8).

Tumor necrosis factor α (TNFα) is a proinflammatory cytotoxic that induces apoptosis in some cell types (15–16). TNFα exerts its effects by binding to TNFα receptors TNFR1 and TNFR2. The cytotoxic effects of TNFα are mediated by TNFR1, which has a cytoplasmic death domain that interacts with an adaptor protein, TNFα-receptor-associated death domain (TRADD), following ligand binding. TRADD subsequently interacts with another adaptor protein, FAS-associated death domain (FADD), which recruits and activates pro-caspase-8, resulting in activation of the cell death machinery.

The keratin family of proteins forms the intermediate filaments of epithelial cells, which are heterodimers of type I (acidic) and type II (neutral basic) keratins. The expression of the various combinations of keratin isomers depends on the pathway of epithelial differentiation; keratin 5 (K5) and K14 heterodimers (K5/K14) and K1/K10 are expressed in basal and suprabasal keratoyctyes of the keratinized squamous epithelium, respectively (17–22). Mutations in either K5 or K14 genes markedly alter tissue integrity and cause epidermolysis bullosa simplex (EBS), a well defined blistering disorder in the epidermis of skin (23). Certain types of EBS are characterized by formation of mutant K5 or K14 aggregates in vivo (24), and transfection of disease-causing mutant keratins results in protein aggregations in vitro (25).

In the present study, we find that transfection of mutant K14 into keratinocytes induces formation of K14 aggregates. We investigated the mechanism linking protein aggregates to cell death using an in vitro cellular EBS model. Our data indicate that autocrine/paracrine TNFR1 stimulation and the resultant caspase-8-mediated apoptosis links keratin aggregates to cell death in keratinocytes.

MATERIALS AND METHODS

Plasmid Construction—cDNA containing the entire coding region of K14 (K14(WT)) was subcloned into the pcDNA 3.1/V5-His vector (Invitrogen). The replacement of Arg125 with Cys (K14(R125C)) was made by overlap extension using PCR methods, and subcloned into the pcDNA3.1/V5-His vector. Pairs of primers used for the first PCR to generate the K14(R125C) fragment were: sense, 5′-ATGACTACCTGCAGCC-3′; antisense, 5′-TGGACGCTGCACCT-3′. The second PCR fragments was verified by the dideoxynucleotide chain termination method using the 377 DNA sequencing system (Applied Biosystems).

Cell Culture and Plasmid Transfection—The culture and transfection of HaCaT cells were carried out as previously described with minor modifications (27, 28). Briefly, cells were plated on 35- or 60-mm dishes at a density of 2 × 10^4 cells/ml 24 h before plasmid transfection, and cultured in Dulbecco’s modified Eagle’s medium (450 mg/dl glucose) supplemented with 10% (v/v) fetal bovine serum. A portion of 2 μg of K14 (WT) or one of the K14 mutants in the pcDNA3.1/V5-His vector for 35-mm dishes and 5 μg for 60-mm dishes were transfected into cells with LipofectAMINE plus reagent (Invitrogen) according to the manufacturer’s instructions. Forty-eight hours after transfection, cells were collected for further analysis. In each experiment, transfection efficiency was confirmed to be similar among types of transfected plasmids using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining (29).

Primary Antibodies—Anti-V5 antibody was purchased from Invitrogen, anti-ubiquitin and anti-TNFα antibody from Dako, anti-cyclin D1 (D9-25), anti-Bip (1:100), anti-cleaved caspase 3 (1:500), anti-cleaved caspase 8 (1:500), anti-ubiquitin (1:20), anti-cleaved caspase 3 (1:300), anti-cleaved caspase 8 (1:300), and anti-cleaved caspase 9 (1:300)) overnight at 4 °C, and detection was with fluorescein isothiocyanate-conjugated antibody to rabbit IgG or a combination of Cy3-conjugated streptavidin and biotin-conjugated anti-mouse IgG. Immunofluorescence images were viewed with a confocal laser microscope (Olympus).

Immunoprecipitation—Immunoprecipitation was performed as described previously (31). HaCaT cells were washed twice with washing buffer (10 mM Tris (pH 7.4) and 0.25% sucrose) and lysed in precipitation buffer (200 mg/ml NaCl, 200 mg/ml KCl, 1150 mg/ml NaH2PO4, 200 mg/ml MgCl2, 1% Triton X-100, 0.1% Na2HPO4, 0.2% Emgeline (Sigma), 5 mM dithiothreitol, 25 μg/ml aprotinin, and 10 μg each leupeptin, and pepstatin) at 4 °C. Cell lysates were presorbed by incubation with 20 μl of protein G-Sepharose for 3 h at 4 °C followed by centrifugation for 1 min at 1,500 g. The supernatant was incubated with 2 μg/ml of anti-V5 antibody overnight at 4 °C. Protein-antibody complexes were washed with the precipitation assay buffer, eluted with Laemmli buffer (consisting of 62.5 μg Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromphenol blue), and subjected to immunoblot analysis.

Immunoblot Analysis—HaCaT cells were lysed in Laemmli buffer (consisting of 62.5 μg Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromphenol blue) on ice for 30 min, cell debris was removed by centrifugation at 15,000 rpm for 1 min, and supernatant (cell lysates) was collected. Protein concentrations in cell lysates were determined using Bradford reagent (Bio-Rad). Cell lysates containing 30 μg of protein were electrophoresed on an SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The membrane was incubated with primary antibody (anti-V5 (1:5000), anti-ubiquitin (1:1500), anti-cyclin D1 (1:200), anti-phospho-c-Jun (1:500), anti-Bip (1:100), anti-α-tubulin (1:200), anti-cleaved caspase 3 (1:500), anti-cleaved caspase 8 (1:500), anti-cleaved caspase 9 (1:500), or anti-TNFα (1:5000) antibody) for 2 h at room temperature, followed by incubation with horseradish peroxidase-conjugated appropriate secondary antibody, and the proteins were detected using an enhanced chemiluminescence system (Amersham Biosciences). The 1:100 dilution of anti-phospho-c-Jun antibody was tried to confirm the manufacturer’s instructions. The protein expression level was quantified by measuring the density of immunoblotting by NIH Image software.

Assay for 20 S Proteasome Activity—Cells were lysed in buffer comprising 10 mM Tris (pH 7.5), 1 mM EDTA, 20% glycerol, 2 mM ATP, 0.5% Triton X-100, 0.1 mM phenylmethanesulfonyl fluoride, 25 μg/ml aprotinin, and 10 μg each of leupeptin and pepstatin on ice for 30 min. Cell debris was removed by centrifugation at 15,000 rpm for 1 min, and supernatant (cell lysates) was collected. Protein concentrations were determined using the bicinchoninic acid assay (Pierce). Cell lysates containing 0.2 mg of protein were incubated with 1 mM carbobenzoxy-L-leucyl-L-leucyl-L-glutamic acid α-4-methylouanylanyl-γ-amide in the presence of 10 μM proteasome activator zLLLal was considered to represent relative 20 S proteasome activity. The sequence of each plasmid construct was verified by the dideoxynucleotide chain termination method using the 377 DNA sequencing system (Applied Biosystems).

Annexin-V and TUNEL Staining—106 cells were washed twice with phosphate-buffered saline comprised of 80 mM Na2HPO4, 20 mM NaH2PO4, and 100 mM NaCl (pH 7.5) and centrifuged at 200 × g for 5 min. Cell pellets were resuspended in phosphate-buffered saline, incubated with fluorescein isothiocyanate-conjugated annexin-V (Roche Applied Science) for 20 min on ice, and supernatant (cell lysates) was fixed with methanol. Cells on glass coverslips were incubated with primary antibodies (anti-V5 (1:3000), anti-ubiquitin (1:200), anti-cleaved caspase 3 (1:300), anti-cleaved caspase 8 (1:300), anti-cleaved caspase 9 (1:300)) overnight at 4 °C, and detection was with fluorescein isothiocyanate-conjugated antibody to rabbit IgG or a combination of Cy3-conjugated streptavidin and biotin-conjugated anti-mouse IgG. Immunofluorescence images were viewed with a confocal laser microscope (Olympus).
FIG. 1. Ubiquitination of K14(R125C) aggregates and their effects on UPS function. a, double stainings of HaCaT cells with 1:300 diluted anti-V5 antibody (red, left panels) and 1:20 diluted anti-ubiquitin antibody (green, middle panels). Right panels show merge of the two stainings. Scale bars: 25 μm. b, cell lysates from HaCaT cells were immunoprecipitated with anti-V5 antibody (2 μg/ml). Protein-antibody complexes were eluted with Laemmli buffer, electrophoresed on a SDS 10% polyacrylamide gel, and immunoblotted with 1:50 diluted anti-ubiquitin antibody. c, density of immunostaining greater than 52 kDa was measured in five experiments, as in a, and was quantified with NIH Image software. d, cell lysates (30 μg of proteins) were electrophoresed on a SDS 10% polyacrylamide gel, and immunoblotted with 1:5000 diluted anti-V5 antibody. e, quantification of the density of immunoblot for K14 (n = 5). f, 20 S proteasome function (n = 4). g, representative immunoblot with 1:200 diluted anti-cyclin D1 antibody of three experiments.

RESULTS

Aggregations and Ubiquitination of Mutant K14—First, to determine whether mutant K14 protein aggregates form in cultured keratinocytes as in keratinocytes of EBS in vivo (24), we transfected HaCaT cells, an epidermis-derived cell line, with V5-tagged K14(WT) or K14(R125C), the most common mutation in the Dowling-Meara type of EBS (25, 33), and stained them with an anti-V5 antibody. Whereas wild-type K14 (K14(WT)) form fine filamentous networks in the cytoplasm (Fig. 1a, upper left panel), K14(R125C) are aggregated, failing to form a coherent filamentous network (Fig. 1a, lower left panel).

Ubiquitination is currently recognized as a signal not only for protein degradation but also for various cell functions including endocytosis, trafficking, and exocytosis (34). Because many protein aggregates that are ubiquitinated are related to the pathophysiology of neurodegenerative diseases (35), we investigated to determine whether mutant K14 aggregates are ubiquitinated. We stained cells transfected with V5-tagged K14(WT) or K14(R125C) dually with an anti-V5 and an anti-ubiquitin antibody. K14(WT)-transfected cells exhibit only weak immunoreactivities for ubiquitin, diffusely throughout the cytoplasm (Fig. 1a, upper middle panel). In contrast, K14(R125C)-transfected cells exhibit strong immunoreactivities for ubiquitin, diffusely throughout the cytoplasm (Fig. 1a, lower middle panel). The V5 immunoreactivities representing K14(R125C) aggregates and strong ubiquitin immunoreactivities overlap, suggesting ubiquitination of K14(R125C) aggregates (Fig. 1a, lower right panel).

To confirm that K14(R125C) is ubiquitinated, lysates of cells transfected with K14(WT) or K14(R125C) were immunoprecipitated with anti-V5 antibody and immunoblotted with anti-ubiquitin antibody (Fig. 1, b and c). In K14(WT)-transfected cells, a weak smear pattern appeared at molecular weights greater than 52,000, the molecular size of K14. When cells were treated with 10 μM zLLLal, an inhibitor of 20 S proteasome, for 3 h prior to cell harvest, the density of smear pattern immu-
nontaining was markedly increased, showing that K14(WT) is under control of the UPS, as has been previously reported (36). In cells transfected with K14(R125C), a smear pattern of ubiquitin immunostaining was evident without treatment with zLAL, and zLAL did not further increase the density.

To ascertain that the UPS is utilized for protein degradation, the expression level of K14 protein was assessed by immunoblot analysis (Fig. 1, d and e). K14(WT) expression is significantly increased by zLAL treatment, whereas K14(R125C) expression is high even without zLAL treatment and not further increased by zLAL. These results indicate that ubiquitination of K14 is utilized as a signal for degradation, conferring resistance of mutated K14 to degradation by the 20 S proteasome.

**K14(R125C) Aggregates Inhibit 20 S Proteasome Function**

To determine whether ubiquitinated mutant K14 aggregates inhibit 20 S proteasome degradation, we first examined the effects of K14(R125C) aggregates on 20 S proteasome function. To measure 20 S proteasome function, the fluorescent UPS substrate z-Leu-Leu-Glu-α-methylcoumaryl-7-amide, which exhibits fluorescence upon its breakdown by 20 S proteasome, was used. Induced fluorescence was significantly attenuated in K14(R125C)-transfected cells compared with K14(WT)-transfected cells (Fig. 1f).

We also tested the effects of K14(R125C) aggregates on turnover of cyclin D1, a defined 20 S proteasome substrate (37) that is not relevant to K14. Immunoblot analysis showed that in K14(WT)-transfected cells, the protein level of cyclin D1 was low in the basal state and markedly increased by treatment with zLAL. In K14(R125C)-transfected cells, the protein level of cyclin D1 was high in the control state, and zLAL treatment did not further increase its expression level (Fig. 1g). These results indicate that K14(R125C) aggregates suppress 20 S proteasome function, interfering with the turnover of proteins such as cyclin D1 that are under the regulated control of the UPS in normal circumstances.

**Apoptosis in K14(R125C)-transfected Cells**—We then investigated the fate of keratinocytes having K14(R125C) aggregates. We utilized fluorescein isothiocyanate-labeled annexin-V to detect apoptotic cells: translocation of phosphatidylserine from the inner leaflet of plasma membrane to the outer leaflet is a biochemical marker of apoptosis that can be detected by the phosphatidylserine-binding protein annexin-V. Fluocytometric analysis showed annexin-V-positive cells at significantly increased by K14(R125C) transfection (47.6 ± 5.2%) but not by K14(WT) transfection (20.1 ± 0.8%), compared with mock transfection (18.7 ± 1.6%)(Fig. 2a). We also performed TUNEL staining in K14(WT)- or K14(R125C)-transfected cells. Positive TUNEL stainings were observed in the nuclei in K14(R125C)-transfected cells but not in K14(WT)-transfected cells (Fig. 2b). These data indicate susceptibility of keratinocytes having K14 mutations to apoptosis.

**Induction of ER Stress**—Protein aggregates are frequently related to ER stress (12, 38, 39). To determine whether K14(R125C) aggregates induce ER stress, we examined the phosphorylation of one of the stress-activated kinases, c-Jun, and the expression level of the unfolding protein, Bip, both of which are biomarkers of ER stress (Fig. 3). Immunoblot analysis with an anti-phospho-c-Jun antibody revealed c-Jun to be considerably phosphorylated in K14(R125C)-transfected cells but only barely in mock- or K14(WT)-transfected cells. The expression level of Bip was significantly increased in cells transfected with K14(R125C) but not in K14(WT)-transfected cells, compared with mock transfection. These data suggest that, as in neurodegenerative diseases, K14(R125C) aggregates inhibit the UPS, subsequently inducing ER stress, resulting in apoptosis.

**Magnitude of Cellular Events in 3 Types of K14 Mutations**—EBs is classified into three types according to the severity of clinical phenotype in decreasing order: Dowling-Meara, Köbner, and Weber-Cockayne. K14(R125C) is the most common mutation in Dowling-Meara (25, 33), K14(V270M) (replacement of Val270 with Met) in Weber-Cockayne (40), and K14(L384P) (replacement of Leu384 with Pro) in Köbner (41). HaCaT cells were transfected with each of K14(WT), K14(R125C), K14(V270M), or K14(L384P) cloned in the pcDNA3.1/V5-His vector. The percentage of cells with aggregates among V5-positive cells were 48.1 ± 4.2, 10.2 ± 2.3, and 20.1 ± 1.4%, in cells transfected with K14(R125C), K14(V270M), and K14(L384P), respectively, significantly higher than in cells transfected with K14(WT) (2.2 ± 0.4%). It is highest in K14(R125C), moderate in K14(L384P), and lowest in K14(V270M) among the three mutants (Fig. 3a). Both suppression of 20 S proteasome activity (Fig. 3b) and induction of ER stress represented by c-Jun phosphorylation and Bip induction (Fig. 3, d and e) were significantly augmented in all of the cells transfected with each mutant, compared with controls of K14(WT) and mock. The magnitude of these cellular events was greatest in K14(R125C). There was no significant difference between K14(L384P) and K14(V270M) (Fig. 3, b, d, and e).

**Activation of Caspases-3 and -8**—To investigate the mechanism linking ER stress to cell death, we first determined which caspases are activated in keratinocytes with K14 aggregates. We immunostained cells dually with anti-V5 antibody and one of the anti-cleaved caspase antibodies (Figs. 4, a–c). Whereas K14(WT)-transfected cells did not show positive immunoreactivities for any of the cleaved caspases, cells with K14(R125C) aggregates exhibited positive immunoreactivities for activated caspases-3 and -8, but not for activated caspase-9.

We also performed immunoblot analyses with anti-cleaved caspase-3, -8, and -9 in K14(R125C)-transfected cells, caspase-3 and -8 but not caspase-9 were activated (Fig. 4d). Because an antibody against cleaved caspase-12 is not available, we performed immunoblot analysis with antibody against non-cleaved caspase-12. In K14(R125C)-transfected cells, an immunopositive band with smaller molecular weight than non-cleaved caspase-12, which would represent cleaved, activated caspase-12, was not detected (Fig. 4d).
We then assessed the expression levels of TNFα in the epidermis (44), the possible involvement of the TNFα pathway in K14(R125C)-induced apoptosis was examined. We first tested the effects of neutralizing TNFα antibody on keratinocyte apoptosis. The addition of TNFα antibody at 0.1 μg/ml to the medium of K14(R125C)-transfected cells partially but significantly decreased the fraction of annexin-V positive cells (from 44.2 ± 3.5% with TNFα antibody, p < 0.01) (Fig. 5a). These data suggest that TNFα is released from HaCaT cells into the culture medium. We then measured the content of TNFα in the medium with a solid phase enzyme amplified sensitivity immunoassay. TNFα was significantly higher in K14(R125C)-transfected cells (104.5 ± 10.5 pg/ml) than in K14(WT) (65.9 ± 5.2 pg/ml) or mock-transfected cells (59.9 ± 5.8 pg/ml) (Fig. 5b).

We then assessed the expression levels of TNFα protein in keratinocytes by immunoblot analysis to be significantly higher in K14(R125C)-transfected cells than in mock- or K14(WT)-transfected cells (Fig. 5c). These results suggest that mutant K14 aggregates stimulate both TNFα synthesis and its release, at least in cultured keratinocytes.

Impairment of Interaction with TRADD in K14(R125C)—K14 and K18 attenuate TNFα-induced cytotoxicity through interaction with TRADD, inhibiting death-inducing signaling complex formation (26, 45). We investigated to determine whether binding to TRADD is modulated in the K14 mutant using the yeast two-hybrid assay. Because the C-terminal region of TRADD (TRADD(CT)) interacts more strongly with K14 than with full-length TRADD (26), yeast Y190 was transfected with various combinations of K14 subcloned into the GAL-4 binding domain vector and C terminus of TRADD (TRADD(CT)) subcloned into the GAL-4 activation domain vector. Transcription of the reporter gene LacZ was assessed qualitatively and quantitatively. Qualitative β-galactosidase assay showed that both yeasts cotransfected with K14(WT) and TRADD(CT) and those with K14(R125C) and TRADD(CT) exhibit positive β-galactosidase activity, indicating direct interaction of both K14(WT) and K14(R125C) with TRADD (Fig. 6a). Yeast with K14(WT) or K14(R125C) and empty GAL4-AD vector or those with TRADD(CT) and empty GAL4-BD vector did not show significant β-galactosidase activity, confirming that these interactions are specific. β-Galactosidase activity appeared to be higher in yeast with K14(WT) and TRADD(CT) than in K14(R125C) and TRADD(CT) (Fig. 6a). To confirm this, we performed a quantitative β-galactosidase assay, which showed the magnitude of β-galactosidase activity to be about 3 times higher in K14(WT)-transfected yeast (15.9 ± 2.5 microunits/A₆₀₀) than in K14(R125C)-transfected yeasts (5.4 ± 1.0 microunits/A₆₀₀) (Fig. 6b).

Stimulation of TNFRI by an Autocrine/Paracrine Mechanism—Binding of TNFα to TNFRI sequentially recruits TRADD, FAS-associated death domain FADD, and procaspase-8, thereby forming the so-called death-inducing signaling complex (42, 43) that activates caspase-8. Because TNFRI is expressed in the epidermis (44), the possible involvement of the TNFα pathway in K14(R125C)-induced apoptosis was examined. We first tested the effects of neutralizing TNFα antibody on keratinocyte apoptosis. The addition of TNFα antibody at 0.1 μg/ml to the medium of K14(R125C)-transfected cells partially but significantly decreased the fraction of annexin-V positive cells (from 44.2 ± 4.1% without TNFα antibody to 31.0 ± 3.5% with TNFα antibody, p < 0.01) (Fig. 5a). These data suggest that TNFα is released from HaCaT cells into the culture medium. We then measured the content of TNFα in the medium with a solid phase enzyme amplified sensitivity immunoassay. TNFα was significantly higher in K14(R125C)-transfected cells (104.5 ± 10.5 pg/ml) than in K14(WT) (65.9 ± 5.2 pg/ml) or mock-transfected cells (59.9 ± 5.8 pg/ml) (Fig. 5b).

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DISCUSSION

Protein aggregate-related disorders are characterized by sequential events including formation of mutant protein aggregates, suppression of UPS function, induction of ER stress, and activation of caspases and resultant apoptotic cell death. In the present study, we show that these events all occur in a cellular model of EBS. Keratinocytes have been found to release TNF-$\alpha$ in response to environmental stresses, including oxidative stress and ultraviolet (UV) radiation (46, 47). Our data indicate that protein aggregations in the cytosol also might be a factor in the stimulation of TNF-$\alpha$ release from keratinocytes. In keratinocytes, TNF-$\alpha$ secretion and the subsequent stimulation of TNFR1 and caspase-dependent apoptosis in an autocrine/para- crine pathway may play an important role in the mechanism linking protein aggregates to cell death.

Cytokines frequently exert their effects in an autocrine/para- crine pathway by acting on the cell itself as well as on neighboring cells. This is the case for TNF-$\alpha$ in some cell types including neurons (48), macrophages (49), and adipocytes (50). In keratinocytes, several cytokines including keratinocyte growth factor (51), interleukin-18 (52), and transforming growth factor-$\beta$1 (53) exhibit their effects in this manner. The present data suggest that TNF-$\alpha$ exerts its cytotoxicity in an autocrine/paracrine manner in keratinocytes having mutant K14 aggregates, because TNF-$\alpha$ protein levels both in cells and in culture medium are increased, and a function blocking anti-TNF-$\alpha$ antibody rescues the keratinocytes from apoptosis in cells transfected with mutant K14. It is intriguing that in yeast, TNF-$\alpha$ itself becomes a factor in the induction of ER stress through activation of RNA-activated protein kinase PKR, which phosphorylates and activates eukaryotic initiation factor-2 $\alpha$ and results in up-regulation of unfolding proteins such as Bip and CHOP. Thus, it is possible that TNF-$\alpha$ contours the positive feedback loop in which ER stress induces the release of TNF-$\alpha$, which in turn induces ER stress.

Keratin 8 (K8) and 18 (K18) are the major keratins in non- cutaneous epithelial cells, forming the intermediate filaments in liver, bowel, and single layered epithelia. K8 and K18 bind to the cytoplasmic domain of TNFR2 (54) and K18 also binds to the C terminus of TRADD (26). By forming these multicomple- xes, K8 and K18 prevent death-inducing signaling complex formation (26, 54), which may protect epithelial cells from TNF-$\alpha$-induced apoptosis. Because K14 binds to the C terminus of TRADD (26), K14 also might have a cyto-protective function against TNF-$\alpha$-mediated cytotoxicity in the skin, as K8 and K18 do in hepatocytes. In the present study, we show that the K14(R125C) mutation decreases the affinity to TRADD to about one-third that of K14(WT), enough to impair cyto-protective function. This concurs with the finding that the region interacting with TRADD is the 1A subdomain of K18, because Arg125, which is responsible for K14(R125C), lies in the region corresponding to the 1A subdomain in K14.

It is not clear if autocrine/paracrine stimulation of TNFR1 is...
root ganglion neurons in a transgenic mouse overexpressing peripherin (62). Interestingly, a large number of activated microglia and astrocytes in this transgenic mouse secrete TNFα, which induces a proinflammatory reaction in the adjacent neurons in a paracrine manner. The addition of neutralizing anti-TNFα antibody prevents apoptosis in these peripherin-overexpressing dorsal root ganglion neurons. A similar mechanism could be involved in the pathology of various aggregate-related neurodegenerative diseases. Further studies of the therapeutic potential of selective antagonists of TNFα action for treatment of EBS are required.

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