hScrib, a human homologue of *Drosophila* neoplastic tumor suppressor, is a novel death substrate targeted by caspase during the process of apoptosis

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hScrib, human homologue of *Drosophila* neoplastic tumor suppressor, was identified as a target of human papillomavirus E6 oncoprotein for the ubiquitin-mediated degradation. Here, we report that hScrib is a novel death substrate targeted by caspase. Full-length hScrib was cleaved by caspase during death ligands-induced apoptosis, which generates a p170 C-terminal fragments in Hela cells. *In vitro* cleavage assay using recombinant caspases showed that hScrib is cleaved by the executioner caspases. DNA damage-induced apoptosis caused loss of expression of full-length hScrib, which was recovered by addition of capase-3 inhibitor in HaCat cells. TUNEL positive apoptotic cells, which were identified 4 h after UV irradiation in HaCat cells, showed loss of hScrib expression at the adherens junction. Mutational analysis identified the caspase-dependent cleavage site of hScrib at the position of Asp-504. Although MDCK cells transfected with GFP-fused wild-type hScrib showed loss of E-cadherin expression and shrinkage of cytoplasm by UV irradiation, cells transfected with hScrib with Ala substitution of Asp-504 showed resistance to caspase-dependent cleavage of hScrib and intact expression of E-cadherin. These results indicate that caspase-dependent cleavage of hScrib is a critical step for detachment of cell contact during the process of apoptosis.

Introduction

Programmed cell death and morphological apoptosis is an intrinsic mechanism of self-destruction of cells (Steller 1995). Well-defined programmed cell death mechanism is crucial for successful embryogenesis and organogenesis (Jacobson *et al.* 1997). Disturbance of the apoptotic functions, which govern homeostasis of tissues, causes proliferation of overgrowing cells and leads to the tumor formation. The apoptotic surveillance mechanism usually causes fragmentation of chromosome and elimination of uncontrolled over-proliferated cells. Cancer cells are considered to develop by escaping from the cellular surveillance mechanisms governing tissue homeostasis. We previously identified hScrib as a target protein of high-risk human papillomavirus (HPV) E6 oncoprotein, which is considered to have a causal role in development of cervical cancer, for the ubiquitin-mediated degradation (Nakagawa & Huibregtse 2000). hScrib is shown to regulate cell cycle progression depending on its association with tumor suppressor protein adenomatous polyposis coli (APC) (Nagasaka *et al.* 2006; Takizawa *et al.* 2006). *Drosophila* Scribble localizes at the septate junction, which is functionally identical to the mammalian tight junction (Bilder & Perrimon 2000). hScrib localizes at the adherens junction in the epithelial MDCK cells (Nakagawa *et al.* 2004). Cysteine proteases from caspase family, which cleave their target proteins exclusively after the Asp residue, have an important function in the process of apoptosis (Brancolini *et al.* 1997; Nicholson & Thornberry 1997; Salvesen & Dixit 1997). Many proteins localized at the adherens junction are targeted by caspase for their cleavage. For instance, β-catenin (Brancolini *et al.* 1997), E-cadherin, plakoglobin, focal adhesion kinase (Levkau *et al.* 1998) and human Discs large (hDlg) (Gregorc *et al.*

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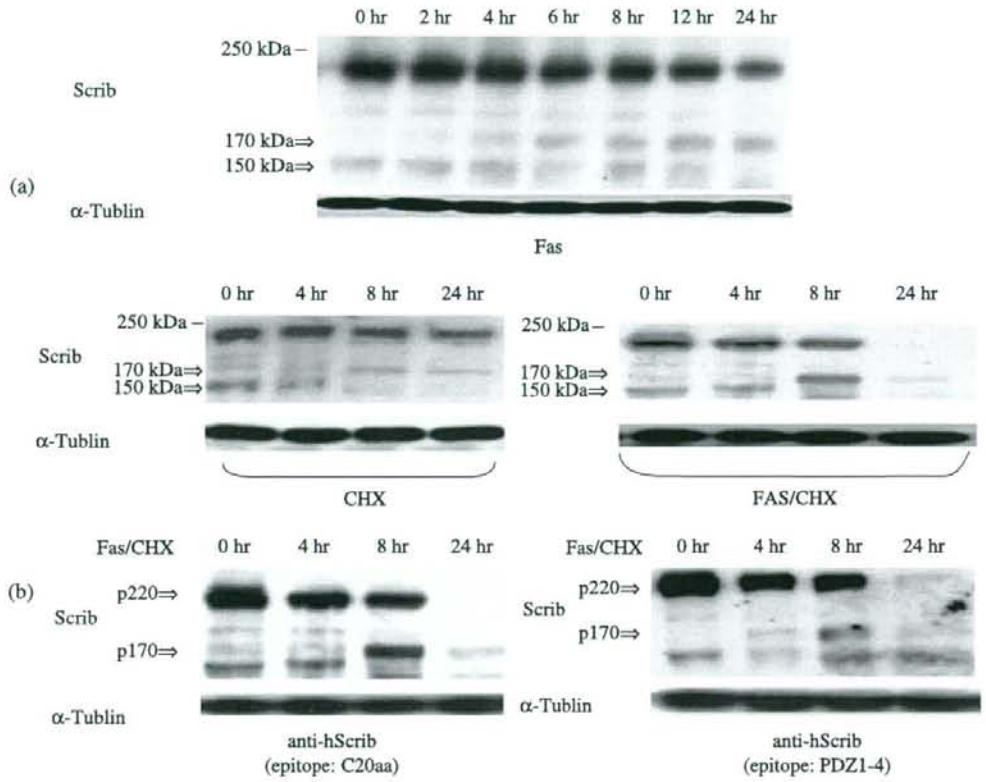


Figure 1 Cleavage of hScrib in Hela cells during Fas ligand-induced apoptosis. (a) HeLa cells were treated with Fas, CHX alone or Fas–CHX. Samples were taken at the times indicated. Western blotting as described in the Material and methods section monitored the amount of hScrib. The expression α-Tublin was shown as the internal control for each lane. (b) The cleavage of hScrib during apoptosis induced by Fas–CHX was analyzed by Western blotting with two antibodies raised against two different hScrib antigens. Note that p220 and p170 hScrib were detected by both of two antibodies. (Correction added after online publication 30 May 2008: This is the corrected version of Figure 1.)

2005) are cleaved by caspase during the process of apoptosis. Here, we identified that hScrib is targeted by caspase for cleavage during the process of apoptosis induced by death ligands and DNA damages.

Results

hScrib is cleaved during the process of apoptosis induced by death ligands and DNA damage

hScrib was shown to be cleaved and generate p170 hScrib during the process of apoptosis induced by death ligand, Fas (Fig. 1a). The band of full-length hScrib p220 and that of p170 generated by induction of apoptosis with Fas ligand and Cycloheximide (CHX) were identified by the two anti-hScrib antibodies raised against different epitopes (Fig. 1b). These data indicate that the full-length hScrib (p220) was cleaved during apoptosis and p170 hScrib was the cleaved product of full-length hScrib. There is one additional band in the lane of 0 h (p150, Figs 1, 2), which also disappeared by Fas–CHX or TNF–CHX treatment. This endogenous p150 was also identified by immunoblotting analysis with two anti-hScrib antibodies (Fig. 1b). The origin of p150 hScrib is currently unknown. The addition of CHX augmented cleavage of hScrib by the induction of apoptosis induced by Fas ligand (Fig. 1a). The hScrib p220 was completely disappeared 24 h after Fas–CHX or TNF–CHX treatment (Figs 1, 2). These data indicate that apoptosis activation by Fas ligand or TNF requires coordinate inhibition of

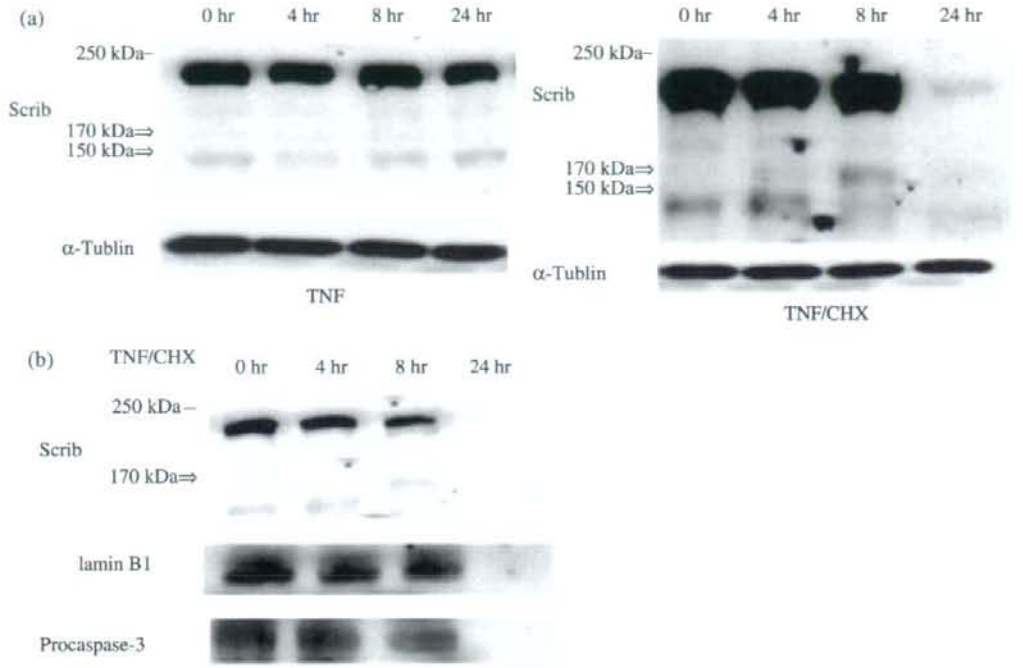


Figure 2 Cleavage of hScrib in Hela cells during TNF-induced apoptosis. (a) HeLa cells were treated with TNF alone or TNF–CHX. Samples were taken at the times indicated and the amount of hScrib was monitored by Western blotting. (b) The efficiency of cleavage of hScrib by the TNF–CHX treatment was compared with that of Lamin B1 or procaspase-3. The efficiency of cleavage of hScrib during apoptosis induced by UV irradiation was equivalent to that of Lamin B1 or procaspase-3. (Correction added after online publication 30 May 2008: This is the corrected version of Figure 2.)

survival signal that inhibit generation of prosurvival factors mediated by NF-κB (Perez & White 2000; Kucharczak *et al.* 2003). The cleavage of hScrib and generation of p170 hScrib were not observed by only TNF ligand, but by the combination of TNF ligand with CHX (Fig. 2a). We compared the efficiency of cleavage of hScrib by the TNF–CHX treatment with that of Lamin B1 or procaspase-3, which are reported to be the early targets for cleavage by caspase during apoptosis (Samejima *et al.* 1999). The efficiency of cleavage of hScrib during apoptosis induced by UV irradiation was equivalent to that of Lamin B1 or procaspase-3 (Fig. 2b).

hScrib localizes at the adherens junction in normal cells as previously reported (Fig. 3a) (Nakagawa *et al.* 2004). DNA damage by UV irradiation caused loss of expression of hScrib at the adherens junction in the apoptotic cells showing condensed (Fig. 3, arrow) or fragmented (Fig. 3, arrow head) nucleus showed by the Hoechst staining.

Loss of expression of hScrib is an early event during apoptosis-induced DNA damage

UV irradiation induced progressive decrease of hScrib expression at the cellular membrane along with time after UV irradiation (Fig. 4). Loss of hScrib expression was identified in the TUNEL positive cells after 4 h of UV irradiation (Fig. 4 UV4 h, arrow indicates TUNEL positive cells, which lost membrane bound expression of hScrib). These data suggest the possibility that hScrib in involved in the cellular detachment at the adherens junction in the early stage of apoptosis. The involvement of hDlg in elimination of apoptotic cell from cellular contact with normal cells, has been reported (Gregorc *et al.* 2005). We analyzed loss of expression of these tumor suppressors in Caco-2 cells, as these tumor suppressors are human homologues of *Drosophila* neoplastic tumor suppressors, in which mutation causes loss of tissue architecture and overgrowth of epithelial cells (Bilder &

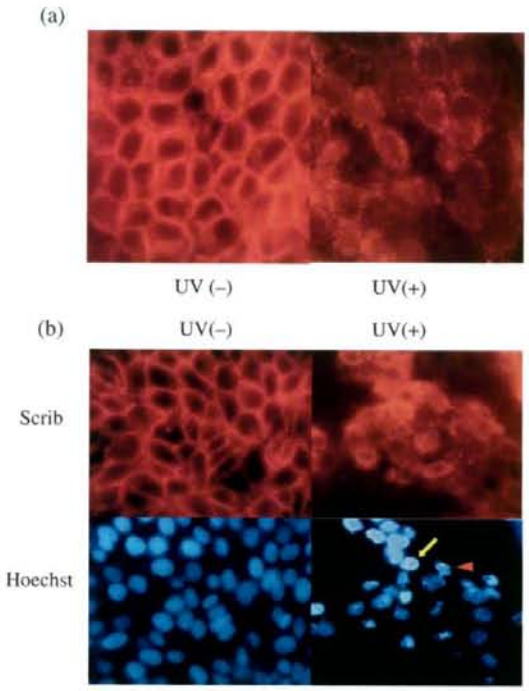


Figure 3 Loss of hScrib expression by the apoptosis induced by UV irradiation in HaCaT cells. (a) Apoptosis was induced with UV irradiation as described in the Materials and methods. Immunofluorescence images with anti-hScrib antibody were taken under the confocal microscopy before and after 6 h of UV irradiation. (b) Loss of hScrib expression at the cellular membrane was observed in the apoptotic cells showing condensed (arrow) or fragmented (arrow head) nucleus showed by the Hoechst staining.

Perrimon 2000). As shown in Fig. 5, the expressed level of hDlg in Caco-2 cells 8 h after UV irradiation was almost equivalent to that of untreated cells (Fig. 5a). In contrast, expression of hScrib was almost diminished in cells after 8 h of UV irradiation (Fig. 5a). Immunofluorescence analysis showed that the membrane-bound expression of hScrib was not observed, and it showed that dot-like expression, whereas hDlg still showed the membrane-bound expression in cells after 6 h of UV irradiation (Fig. 5b). At 12 h after irradiation of UV, expression of hScrib was not observed, but hDlg showed still faint but membrane-associated expression (Fig. 5b). These results indicate that hSrib is targeted by proteolysis earlier than the hDlg in apoptotic cells induced by UV irradiation.

hScrib is cleaved by the executioner caspases *in vitro*

Based on data described above, we investigated the possibility that hScrib is targeted for cleavage by caspase. *In vitro* translated hScrib was incubated with recombinant caspase-3, caspase-6, caspase-7 or caspase-8. hScrib was cleaved *in vitro* by the executioner caspases, caspase-3, caspase-6 and caspase-7 (Fig. 6a). The *in vitro* cleavage of hScrib by caspase-3 was completely repressed by the caspase-3 inhibitor (Fig. 6b). These data indicate that hScrib is a novel death substrate targeted by the executioner caspases.

hScrib is targeted by executioner caspase for proteolysis during the process of apoptosis

Next, we investigated the possibility that hScrib is targeted for cleavage by caspase *in vivo* during apoptosis. Apoptosis induced by UV irradiation caused cleavage of hScrib as mentioned above (Fig. 7). Loss of expression of hScrib as a result of cleavage by caspase activated with UV irradiation was recovered by addition of the caspase-3 inhibitor in the medium (Fig. 7). The caspase-6 inhibitor also showed the repressive effect on the cleavage of hScrib during apoptosis, but its inhibitory effect was weaker than that of caspase-3 inhibitor (Fig. 7). These data indicate that hScrib is targeted for cleavage during the process of apoptosis mainly by caspase-3.

504 Asp of hScrib is critical for caspase-dependent cleavage

Asp-X-X-Asp is reported to be conserved motif for caspase-dependent cleavage (Nicholson & Thornberry 1997; Talanian *et al.* 1997). There are two putative caspase-dependent cleave sites with amino acids Asp-X-X-Asp at 1068 Asp-X-X-1071 Asp and at 1131 Asp-X-X-1134 in amino acids sequence of hScrib. Alanine substitution of these Asp amino acids did not allow hScrib to resist to caspase-dependent cleavage (data not shown). hScrib consists of 16 canonical Leucine rich repeats (LRRs), LAP specific domain-a (LAPSD-a), LAPSD-b and four PDZ domains. We investigated which domains of hScrib are susceptible for cleavage by caspase *in vitro* by using several hScrib C-terminal deletion mutants. Human Scrib mutants, LRR–PDZ3, LRR–PDZ2 and LRR–PDZ1 were cleaved by recombinant caspase-3, whereas LRR–LAPSD and LRR were not susceptible for cleavage. These data indicate that hScrib is cleaved by caspase-3 at the C-terminal region of LAPSD (Fig. 8a). Mutational analysis found that Ala substitution of Asp 504 allows hScrib to resist to caspase-3 dependent cleavage (Fig. 8b).

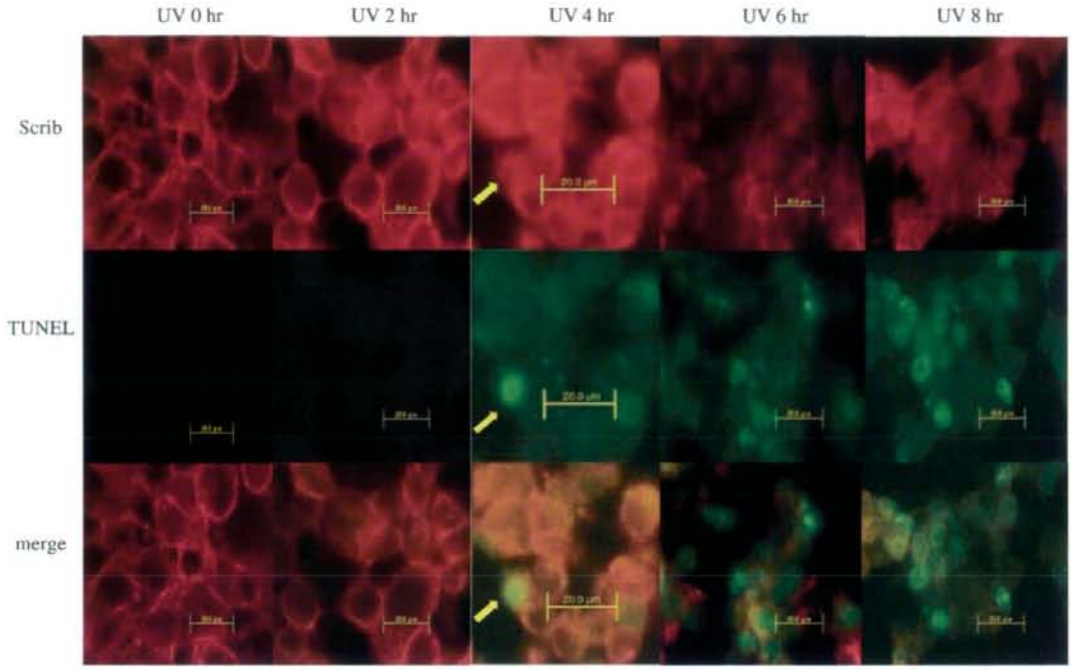


Figure 4 Loss of hScrib expression is an early event during apoptotic process. Cells irradiated with UV were analyzed by immunofluorescence staining with anti-hScrib antibody and TUNEL at the indicated time after UV irradiation. Note that hScrib expression was lost in the TUNEL positive cell (UV 4 h). The arrow indicates that loss of hScrib expression is observed in the TUNEL positive cells after 4 h of UV irradiation.

Alanine substitution of Asp 526 resided at the C-terminus of the LAPD of hScrib did not render hScrib resistant to caspase-3 dependent cleavage (Fig. 8b).

hScrib with Ala substitution of Asp 504 is resistant to caspase-dependent cleavage during the process of apoptosis induced by UV irradiation

GFP-tagged wild-type hScrib transfected MDCK cells showed loss of E-cadherin and hScrib expression at the membrane, fragmentation of nucleus and shrinkage of cytoplasm by apoptosis induction with UV irradiation (Fig. 9a,b). In contrast, GFP-tagged Asp504Ala hScrib mutant transfected MDCK cells showed the intact expression of transfected hScrib and E-cadherin, despite of fragmentation of nucleus showed by the Hoechst staining (Fig. 9c,d). Immunoblotting analysis confirmed that over-expressed GFP–hScrib D504A mutant is resistant to caspase-dependent cleavage activated by apoptosis induction with UV irradiation (Fig. 10a). To quantify the effect of WT and Asp504Ala hScrib mutant on apoptosis induction and cellular detachment, 300 MDCK cells transfected with control vector, GFP–WT hScrib vector, or GFP–hScrib D504A mutant vector were analyzed for apoptosis signals and cellular detachment by Hoechst staining and immunofluorescence analysis of E-cadherin, respectively (Fig. 10b). UV irradiation induced apoptosis and cellular detachment approximately in 80% MDCK cells transfected with control vector or GFP–WT hScrib vector. Although apoptosis was observed over 80% of cells transfected with GFP–hScrib D504A mutant vector, cellular detachment was only observed in 9% of those cells. These data indicate that caspase-dependent cleavage of hScrib has a crucial role in cellular detachment during progression of apoptosis.

Effect of HPV E6 expression on the caspase-dependent cleavage of hScrib during apoptosis

We investigated whether E6 expression have an effect on caspase-dependent cleavage of hScrib. As shown in Fig. 11, hScrib expression level in cells transfected with E6

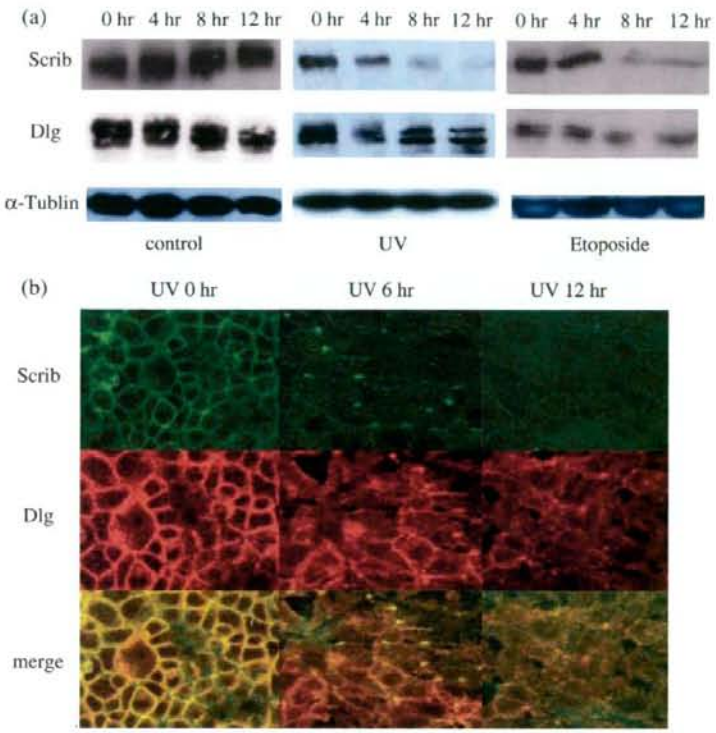


Figure 5 Loss of hScrib expression is earlier event in progressive apoptosis than that of hDlg. Expression of human homologues of *Drosophila* neoplastic tumor suppressor proteins, hScrib and hDlg, was analyzed by the Western blotting (a) and immunofluorescence staining (b) with anti-hScrib and anti-hDlg antibodies during progression of apoptosis. Both of assays indicate that hScrib is targeted by proteolysis earlier than hDlg in the progression of apoptosis induced by UV irradiation.

expression vector was lower than that in cells transfected with control vector, which is consistent with the previous report (Nakagawa & Huibregtse 2000). After induction of apoptosis, caspase-dependent cleavage of hScrib was more evident in cells transfected with control vector comparing with that in cells transfected with E6 expression vector (Fig. 11). The p220 hScrib was not observed in cells transfected with control vector and in those transfected with E6 expression vector after 24 h of UV irradiation, suggesting the possibility that E6 protein expression partially inhibit caspase-dependent cleavage of hScrib.

Discussion

Tissue homeostasis is kept in normal epithelial cells under the surveillance of programmed cell death mechanism (Igney & Krammer 2002). Over-proliferated or over-damaged cells are eliminated by the self-destruction mechanism called apoptosis (Steller 1995; Thompson 1995; Song & Steller 1999). Disruption of intrinsic cell–cell contact is a critical step in the process of apoptosis (Rosenblatt *et al.* 2001). A proteolytic cascade mediated by the caspases family of cysteine proteinases, which specifically cleave target proteins after aspartate residues, has a central role in cell death machinery (Brancolini *et al.* 1997). A number of proteins localized at the adherens and tight junctions have been reported to be targeted by caspases, including E-cadherin, β-catenin, FAK, PAK2, fodrin, plakoglobin, hDlg, focal adhesion kinase, ZO-1, ZO-2, occludin, MAGI-1 and MAGI-2 (Rudel & Bokoch 1997; Wen *et al.* 1997; Janicke *et al.* 1998; Levkau *et al.* 1998; Steinhusen *et al.* 2000, 2001; Bojarski *et al.* 2004; Gregorc *et al.* 2005, 2007; Ivanova *et al.* 2007). We have identified that hScrib is targeted for cleavage by executioner caspase activated by death ligands TNF-α and FAS ligands and UV irradiation. These data indicate that caspase-dependent cleavage of hScrib is a general event in apoptosis.

hScrib is human homologue of *Drosophila* tumor suppressor protein Scribble (Nakagawa & Huibregtse 2000). In *Drosophila*, three tumor suppressor genes (TSGs) *lgl*, *dlg* and *scrib* are categorized as neoplastic TSGs, in which mutation causes loss of apico-basolateral cellular polarity and tissue architecture and simultaneously induces extensive over proliferation in epithelia and neuroblasts (Bilder *et al.*

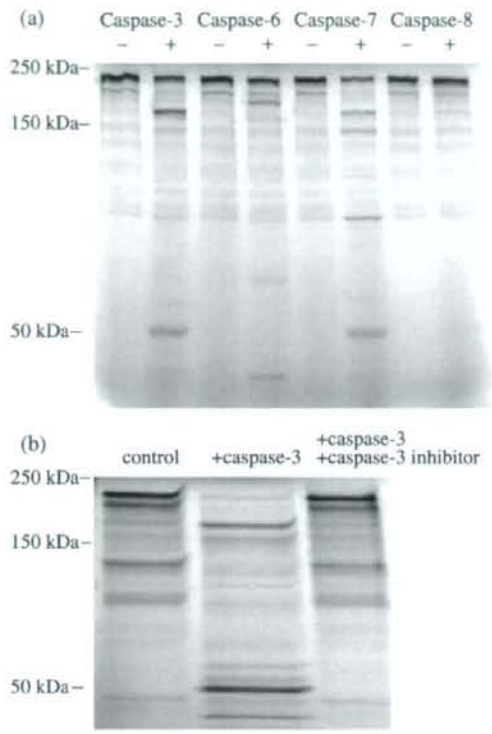


Figure 6 *In vitro* cleavage of hScrib with the executioner caspases. (a) *In vitro* translated [^{35}S] methione-labeled hScrib was incubated with recombinant caspase-3, caspase-6, caspase-7 and caspase-8 as described in the Materials and methods. Cleavage of hScrib by the executioner caspases was confirmed by the SDS electrophoresis and autoradiography. (b) The *in vitro* cleavage of hScrib by caspase-3 was completely repressed by the presence of caspase-3 inhibitor.

2000; Bilder 2003, 2004; Humbert *et al.* 2003; Hariharan & Bilder 2006). The *scrib* mutant clones proliferate, but these excess cells are eliminated by JNK-dependent apoptosis (Brumby & Richardson 2003; Pagliarini & Xu 2003; Tapon 2003). Loss of *scrib* mutation in activated Ras-expressing cells disrupted the epithelial structure of the eye imaginal disc and led to progressive invasion into neighboring structure (Pagliarini & Xu 2003). These data suggest the possibility that disruption of tissue polarity by loss of hScrib is involved in human carcinogenesis in concert with activated expression of oncogenic Ras. hScrib has been shown to be a functional homologue of the *Drosophila* Scribble (Dow *et al.* 2003). hScrib can rescue loss of polarity and inhibit tumorous overgrowth of *scrib* mutant *Drosophila* (Dow *et al.* 2003). Mammalian Scribble was shown to have crucial role in promotion of cell polarity in migrating astrocyte and epithelial cells (Osmani *et al.* 2006; Dow *et al.* 2007).

hScrib localizes at the adherens junction in normal epithelial cells and its expression is down-regulated in the precursor lesions and invasive cancers in the uterine cervix and colon (Nakagawa *et al.* 2004; Gardiol *et al.* 2006). Loss of hScrib expression was observed at the early stage of apoptosis identified by the positive TUNEL signal. hDlg is human homologue of *Drosophila* neoplastic tumor suppressor protein Discs large and is targeted for ubiquitin-mediated degradation by the high-risk HPV E6 protein (Gardiol *et al.* 1999). We analyzed loss of expression of these human homologues of *Drosophila* neoplastic tumor suppressor proteins during apoptosis and found that loss of hScrib expression is earlier event than that of hDlg. These data indicate that proper expression of hScrib is essential for construction of adherens junction and elimination of hScrib expression is also crucial for the disruption of junctional protein complex in damaged cells during apoptosis.

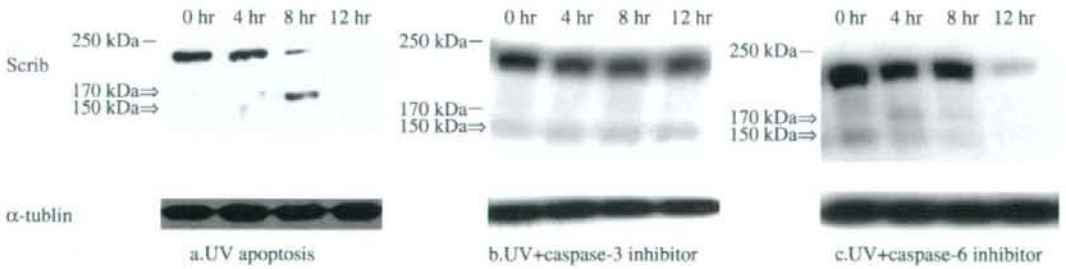


Figure 7 *In vivo* cleavage of hScrib by caspase-3 and caspase-6. The hScrib expression was lost by the irradiation of UV. The lost of hScrib expression after the apoptosis induction was inhibited by the presence of the inhibitor of executioner caspases, especially by the caspase-3 inhibitor. Note that generation of p170 hScrib was repressed by the caspase-3 inhibitor, but not by the caspase-6 inhibitor.

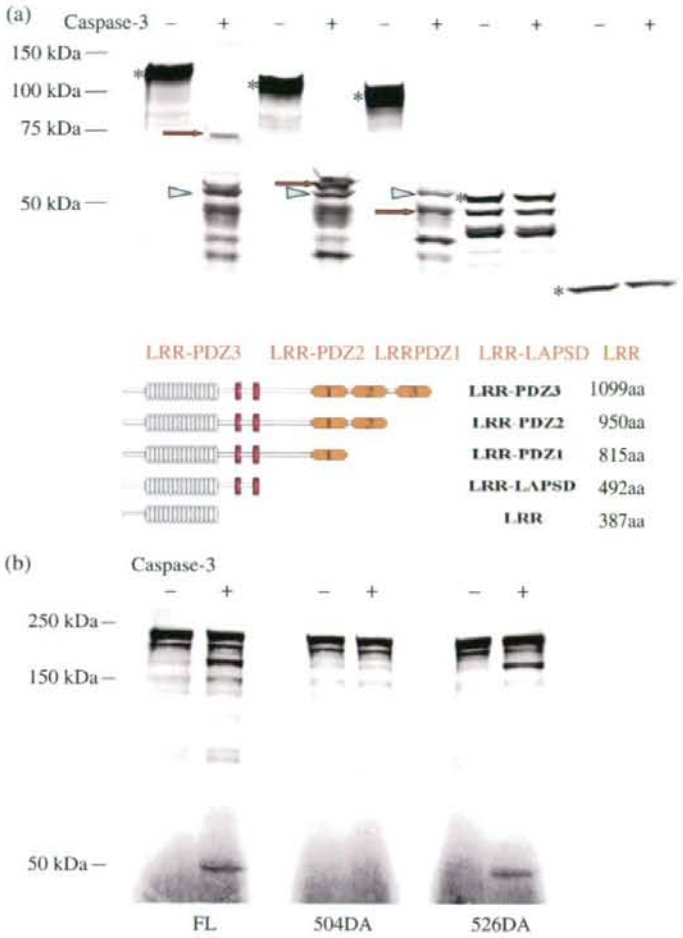
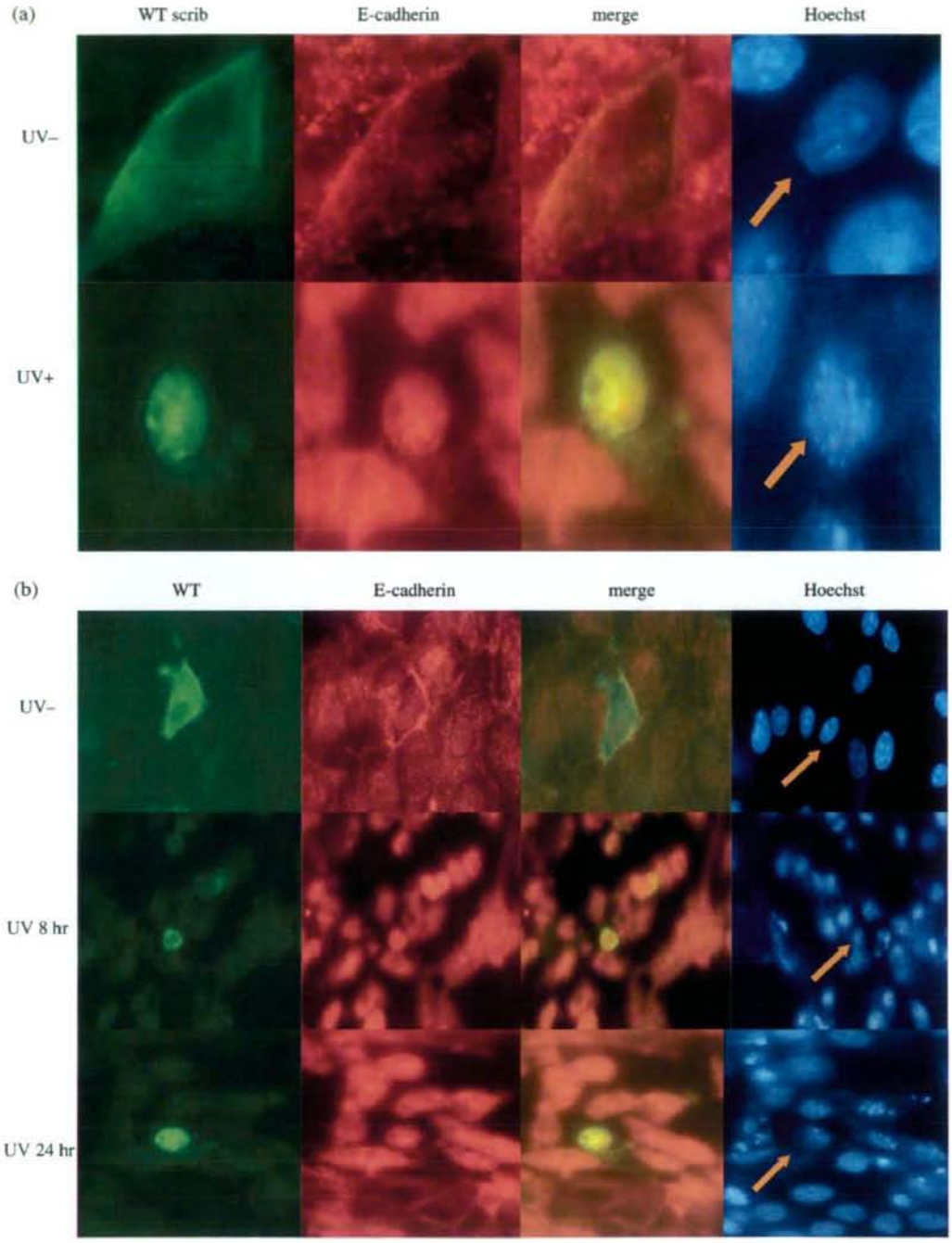
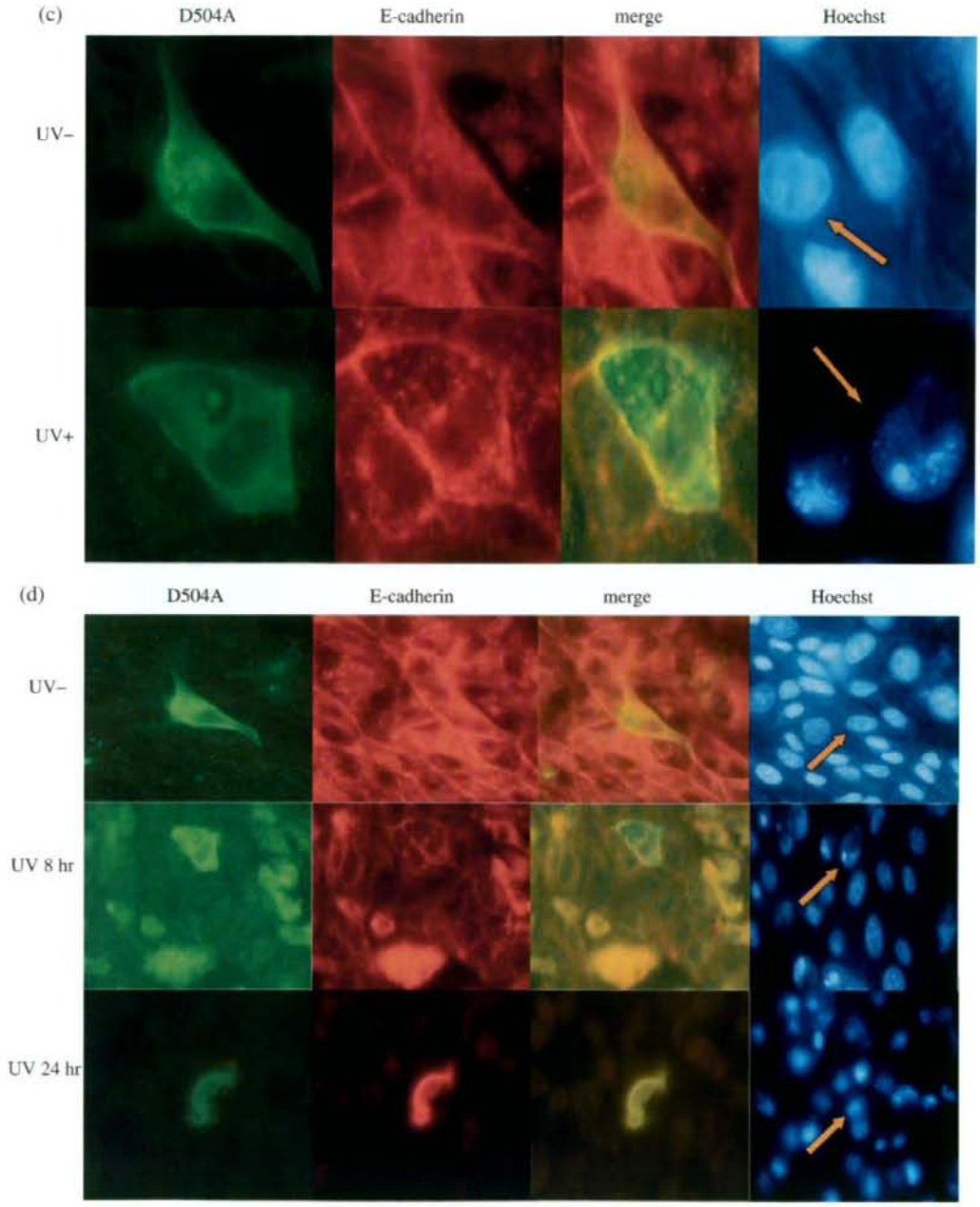


Figure 8 Analysis of cleavage site of hScrib by the caspase-3. (a) *In vitro* translated [^{35}S]-methione-labeled hScrib deletion mutants were incubated in the presence of recombinant caspases-3. The Scheme of hScrib C-terminal deletion mutants is shown in Fig. 8a. Human Scrib mutants, LRR–PDZ3, LRR–PDZ2 and LRR–PDZ1 were cleaved by recombinant caspase-3, whereas LRR–LAPSD and LRR were not susceptible for cleavage by caspase-3. Note that the protein band with same molecular weight (marked by the blue arrow head), which is considered to be the N-terminal part of proteins cleaved by caspase-3, is seen in cleaved LRR–PDZ3, LRR–PDZ2 and LRR–PDZ1 (Fig. 8a). The full-length translated hScrib mutants were indicated by the asterisk. Several fragments with much smaller sizes are seen for translated hScrib deletion mutants, especially for LRR–LAPSD. The protein bands, which are considered to be the C-terminal part of LRR–PDZ3, LRR–PDZ2 and LRR–PDZ1 hScrib mutants cleaved by caspase-3 are indicated by the red arrows. (b) Wild-type hScrib and Alanine substitution hScrib mutants of Asp 504 (D504A) and Asp526 (D526A) were tested for *in vitro* cleavage in the presence of recombinant caspases-3. Human Scrib D504A mutant is resistant to caspase-3 dependent cleavage.

We screened hScrib amino acids sequence for the potential cleavage site by caspase and found two D-X-X-D sequences (D_{1068}-V-R-D_{1071} and D_{1131}-P-T-D_{1134}), which are typical caspase-3 recognition sequences (Talanian *et al.* 1997). None of single amino acid substitution of these four Asp residues rendered hScrib resistant for caspase-dependent cleavage (K. S. and S. N. unpublished data). hScrib is a member of LAP (LRRs and PDZ domains) proteins. It has 16 canonical LRRs at the N-terminal region and four copies of the PDZ domain in its C-terminus (Santoni *et al.* 2002). Between these structures lies a 38-amino acid LRR-like domain called LAPSD-a. A second conserved sequence specific to LAP proteins and unrelated to LRR motifs between LRRs and PDZ domains resides at the downstream of LAPSD-a and is named as LAPSD-b. We investigated the region responsible for caspase-dependent cleavage by using deletion mutants of hScrib and found that PDZ domains are not targeted for cleavage by caspase-3 and that the

Figure 9 GFP-fused wild-type hScrib and hScrib D504 mutant were transfected in to MDCK cells. Apoptosis was induced with UV irradiation 48 h post-transfection. Cells were analyzed for the immunofluorescence staining of E-cadherin. Hoechst staining was carried out for the analysis of nuclear fragmentation. The expression of hScrib and E-cadherin was lost in the wild-type hScrib transfected cells (arrow), which show the fragmented nucleus, after induction of apoptosis. Note that expression of E-cadherin is intact as a control in cells transfected with hScrib D504A mutant (arrow), which is resistant for caspase-dependent cleavage, whereas nucleus shows typically apoptotic signal with condensed fragmentation.





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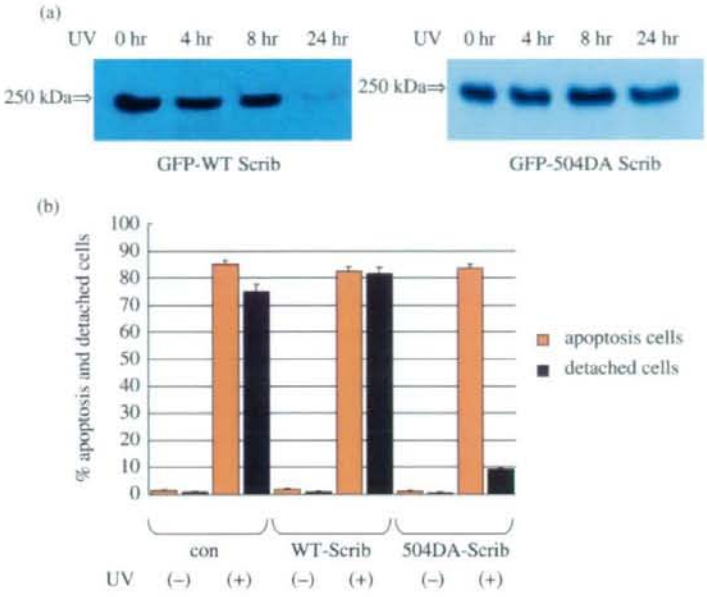

Figure 10 Human Scrib D504A mutant is resistant to caspase-dependent cleavage induced by apoptosis. (a) Immunoblotting analysis confirmed that over-expressed GFP–hScrib D504A mutant is resistant to caspase-dependent cleavage activated by apoptosis induction with UV irradiation. (b) To quantitate the effect of WT and Asp504Ala hScrib mutant on apoptosis induction and cellular detachment, 300 MDCK cells transfected with control vector, GFP–WT hScrib vector, or GFP–hScrib D504A mutant vector were analyzed for apoptosis signals and cellular detachment by Hoechst staining and immunofluorescence analysis of E-cadherin, respectively. The ratio of apoptosis positive cells and detached cells as evidenced by loss of E-cadherin in 300 cells transfected with each vector was analyzed for absence and presence of UV irradiation and shown as histograms.

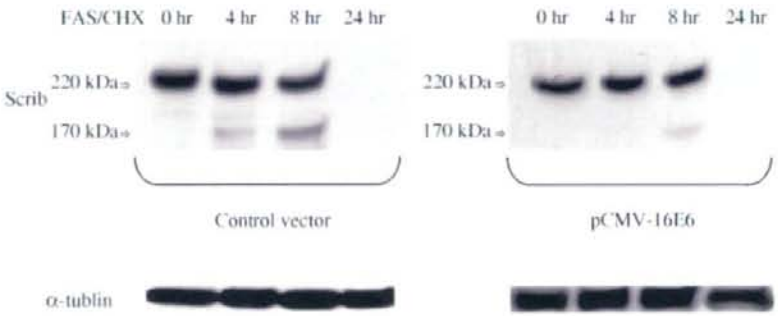

Figure 11 The effect of HPV E6 on the caspase-dependent cleavage of hScrib. Apoptosis was induced by UV irradiation at 48 h post-transfection of control vector or E6 expression vector. Prior the apoptosis induction, hScrib expression level was lower in cells transfected with E6 expression vector. After induction of apoptosis, caspase-dependent cleavage of hScrib was more obvious in cells transfected with control vector as evidenced by the generation of p170 at 4 h post-UV irradiation comparing with that in cells transfected with E6 expression vector. Note that p170 was not observed in cells transfected with E6 vector after 4 h of UV irradiation.

amino acids sequence between LRRs and PDZ domain 1 is potential caspase-dependent cleavage site. The site-specific mapping of a critical amino acid for the caspase-dependent cleavage of hScrib with mutagenesis showed that first ASP residue (amino acid 504) in the region between LAPSD-b and PDZ domain 1 is targeted for cleavage by caspase-3. hScrib N-terminal region containing LRRs, LAPSD-a and LAPSD-b ($hScrib_{1-724}$ and $hScrib_{1-518}$) is reported to localize at the basolateral epithelial membrane (Navarro *et al.* 2005). Our previous study showed that $hScrib_{1-495}$ localizes in the cytoplasm, not at the membrane (Nagasaka *et al.* 2006). It is possible that the cleaved hScrib at amino acid 504 by caspase-3 ($hScrib_{1-504}$) does not target the basolateral membrane. For *Drosophila* Scribble, multi-step localization through LRRs and PDZ domains are necessary for establishment of cortical polarity (Albertson *et al.* 2004; Zeitler *et al.* 2004). hScrib has been shown to be involved in polarity

control in migrating cells by interacting βPIX exchange factor and APC (Audebert *et al.* 2004; Takizawa *et al.* 2006; Dow *et al.* 2007). hScrib have been reported to interact with ZO-2 and zyxin-related proteins, Lipoma Preferred Partner (LPP) protein and TRIP6, at epithelial cellular junctions through its PDZ domains (Metais *et al.* 2005; Petit *et al.* 2005a,b). The caspase-3 dependent cleavage of hScrib at amino acid 504 might disrupt these protein complexes formations at the epithelial cellular junctions through its PDZ domains. The resistance to distraction of adherens junction in the apoptosis-induced epithelial cells transfected with hScrib D504A mutant indicates that caspase-3 dependent cleavage of hScrib is a critical step for elimination of dying cell from normal cells. Our analysis of the effect of E6 expression on the caspase-dependent cleavage of hScrib indicated the possibility that E6 partially inhibit the cleavage. These data suggest the possibility that E6 render some cellular fractions of hScrib resistant to the caspase-dependent cleavage. Further investigations would be required to show comprehensive mechanisms underlying the partial inhibition of caspase-dependent cleavage of hScrib by E6 protein.

In summary, we found that hScrib, which has a fundamental role in tissue polarity architecture, is a novel death substrate targeted by caspase-3. The caspase-dependent cleavage of human homologues of *Drosophila* neoplastic tumor suppressors, hScrib and hDlg, is considered to be an essential step in the elimination of apoptotic cells from the surrounding healthy cells.

Experimental procedures

Tissue culture and apoptosis induction

Human HaCat, CaCo-2 cells and Hela cells were grown in DMEM supplemented with 10% fetal bovine serum. Before induction of apoptosis, cells were plated onto 10-cm dishes and allowed to reach to the confluency. Apoptosis was induced by irradiating UV light (0.24 J) or adding 200 nM etoposide (Sigma, St Louis, MO), 500 ng/mL anti-Fas (MBL, Nagoya, Japan), 100 μg/mL Cycloheximide (CHX) (Sigma) and/or 2000 U/mL TNF (Relia Tech GmbH, Braunschweig, Germany) into the medium. In an additional experiment using caspase inhibitors, 50 μM Z-DEVD-FMK (R&D systems, Minneapolis, MN or Ac-VEID-CHO (Biomol, Pennsylvania, PA) was added into the medium and apoptosis was induced as described above.

Western blotting

Following apoptosis induction, cells were harvested at the indicated hours after induction of apoptosis. The protein concentration of the samples was equalized and samples were analyzed by electrophoresis on 6% SDS PAGE. Levels of hScrib and hDlg protein were determined by Western blotting using ECL advance Western blotting Detection Kit (GE Healthcare Bio-science, Piscataway, NJ) according to the manufacture's instructions. The expression of hScrib was detected using the anti-hScrib goat monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or the anti-hScrib polyclonal antibody raised in rabbit against its PDZ domains as an antigen. The expression of hDlg was detected using the anti-hDlg mouse monoclonal antibody. (Santa Cruz Biotechnology). The expression of Lamin B1 was detected using the anti-Lamin B1 mouse monoclonal antibody. (Santa Cruz Biotechnology). The expression of procaspase-3 was detected using the anti-caspase-3 mouse monoclonal antibody. (Santa Cruz Biotechnology). The expression of GFP–Scrib was detected using the anti-GFP mouse monoclonal antibody (Zymed, San Francisco, CA).

Fluorescence microscopy

HaCaT and CaCo-2 cells were grown overnight on cover slips before induction of apoptosis. Cells were washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde in PBS for 30 min at the times indicated, followed by permeabilization with 0.2%(v/v) Triton X-100 in PBS for 5 min. After extensive washing with 1% BSA–PBS, the cells were incubated with anti-hScrib antibody diluted 1 : 400, and anti-hDlg diluted 1 : 100 in PBS for 60 min. Following an additional round of wash with PBS containing 1% BSA, cells were incubated with donkey anti-goat and rabbit anti-mouse Allexa488 and 568 conjugated antibodies (Invitrogen, Eugene, OR) for 60 min. Expression of protein was investigated under the confocal fluorescence microscopy.

To analyze apoptosis signal, cells were incubated with Hoeshst33342 (Sigma) for 7 min, washed in PBS with 1% BSA, and then mounted on slides.

MDCK cells were transfected with GFP-tagged human scribble constructs, using the PolyFect Transfection Reagent (Qiagen, Hiden, Germany) or Effectene Transfection Reagent (Qiagen) according to manufacturer's instructions. To see the effect of HPV E6 on the caspase-dependent cleavage of hScrib during apoptosis, 293T cells were transfected with HPV E6 expression plasmid (Nakagawa & Huibregtse 2000).

Apoptosis is induced 48 h post-transfection with UV irradiation. At the indicated hours, cells were collected and treated as described above or stained with anti-E-cadherin antibody (BD Transduction Laboratories, Franklin Lakes, NJ) and Alexa568 conjugated anti-mouse antibodies (Molecular Probes, Eugene, OR). In addition, Hoeshst33342 was used to stain the nuclei. Morphological changes of cells induced of apoptosis were monitored using confocal fluorescence microscopy. To quantify the effect of WT hScrib or hScrib mutant D504A on cellular detachment during apoptosis, number of cells showing apoptosis (fragmentation of nucleus, and shrinkage of cytoplasm) and cellular detachment (loss of E-cadherin) were analyzed in 300 MDCK cells transfected with control vector, GFP–WT hScrib, or GFP–hScrib D504A mutant.

In vitro translation of proteins

Proteins were expressed using the Promega TNT coupled transcription-translation Rabbit-Reticulocyte lysate system (Promega,

Madison, WI) according to the manufacture's instructions and radio-labeled with [^{35}S]-methionine (PerkinElmer, Waltham, MA).

Caspase cleavage assays

For *in vitro* caspase cleavage assay, *in vitro* translated hScrib labeled with [^{35}S] methione was incubated in the presence of recombinant caspase-3 (Chemicon, Temecula, CA), caspase-6 (Alexis, Lausen, Switzerland), caspase-7 (Chemicon) or Caspase-8 (BioVision, San Francisco, CA) at 37 °C for 1 h. The reaction was terminated by the addition of SDS loading buffer and boiling. The reaction mixtures were analyzed by SDS-PAGE and autoradiography.

Plasmids

For *in vitro* expression, the cDNA for Scrib was subcloned into the BamHI/NotI sites of pCDNA3. The Scrib Ala substitution mutants of Asp were constructed using overlap polymerase chain reaction (PCR) with Scrib cDNA as a template using the following primers:

5′-CCTTGCCAGCCA***GCC***TCTGGGTCGCCC-3′ (Asp504Ala)

5′-GGCCTGAGTGAA***GCC***TCTCGCCCATCTGCC-3′ (Asp526Ala)

5′-GTGAACGGGCAA***GCC***GTGCGGGATGCC-3′ (Asp_{1068}Ala)

5′-CAAGACGTGCGG***GCT***GCCACGCACCAAG-3′ (Asp_{1071}Ala)

5′-GGCAACCCCCGC***GCC***CCCACAGACGAG-3′ (Asp_{1131}Ala)

5′-CGCGACCCCACA***GCC***GAGGGCATCTTC-3′ (Asp_{1134}Ala)

To generate the deletion mutants of hScrib, the following cDNA sequences were amplified with polymerase chain reaction (PCR) and subcloned into pCDNA3: LRR + PDZ 1–3 (amino acids 1–1096); LRR + PDZ 1–2 (amino acids 1–953); LRR + PDZ 1a (amino acids 1–819); LRR + PDZ 1b LRR + LAPSDb (amino acids 1–495).

For GFP fusions wild type and mutant *human scribble* cDNA were cloned into the HindIII/EcoRI sites of pEGFP–C1 vector.

TUNEL assay

Human HaCaT cells were grown overnight on cover slips before induction of apoptosis.

After induction of apoptosis, cells were washed with PBS and fixed with 3.7% paraformaldehyde in PBS for 30 min, followed by permeabilization with 0.2% (v/v) Triton X-100 in PBS for 5 min.

The TUNEL assay was carried out using Promega Dead-END™ Fluorometric TUNEL System (Promega) according to the manufacture's instructions.

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Oral pilocarpine (5 mg t.i.d.) used for xerostomia causes adverse effects in Japanese

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Abstract

Objective: To evaluate Japanese tolerability to pilocarpine of 5 mg t.i.d.
Methods: From January 2006 to July 2006, 39 patients with xerostomia received 5 mg t.i.d. pilocarpine for at least for 12 weeks unless they had experienced unacceptable adverse effects. All patients received radiotherapy that included the parotid glands in the radiation field >50 Gy. The body weights of the patients ranged from 42 to 73 kg (median 60 kg).
Results: Thirty-six of the 39 patients were evaluable. The tolerated rate was only 47%. Of the 25 patients whose body weights were less than 65 kg, the tolerated rate was 36%, whereas the rate of the 11 patients whose body weights were 65 kg or above was 72% ($p = 0.050$). The most common adverse effect was sweating with an incidence of 64%. Response rate, which was defined as the total number of patients with an increase of at least 25 mm from the baseline in the VAS score divided by the number of maintaining patients among those who started pilocarpine after more than 4 months from the start of radiotherapy, was 40% at 12 weeks ($n = 15$).
Conclusion: For Japanese, 5 mg t.i.d. pilocarpine caused a high incidence of unacceptable adverse effects. A lower dose of pilocarpine needs to be considered.

Keywords: Pilocarpine; Xerostomia; Japanese; Tolerability; Sweating; Radiotherapy

1. Introduction

Lifelong xerostomia associated with salivary dysfunction is a most unpleasant adverse effect resulting from high dose irradiation delivered to the head and neck region [1–5]. Patients with xerostomia experience significant oral discomfort and difficulties in speaking, swallowing, and sleeping [6–9]. These conditions can lead to severe oral disease, nutritional deficiencies and marked decline in quality of life [10].

Treatment of xerostomia is difficult, and previous treatments have included saliva substitutes, hard candy, antimicrobial rinses, and fluoride treatments, all of which have generally been inadequate. Pilocarpine hydrochloride, however, has been approved for effective treatment of radiation-induced xerostomia in many countries. It is a naturally occurring alkaloid that has a broad range of pharmacologic effects, including increasing secretion from the exocrine glands (sweat, salivary, lacrimal, gastric, pancreatic, and intestinal glands). The clinical efficacy of 5–10 mg pilocarpine three times per day (t.i.d.) daily to reduce the symptoms of xerostomia has been studied in several trials in the Western countries [8–18].

On the other hand, pilocarpine is known to cause various kinds of adverse effects expected for a cholinergic agonist (e.g., sweating, rhinitis, nausea, urinary frequency)

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[8,11,12]. The severity of these adverse effects is thought to be dose related. According to Rieke et al. pilocarpine's adverse effects were considered acceptable by patients taking 5 mg t.i.d. [11].

Pilocarpine has been available for clinical use from October 2005 in Japan. However, a study has not yet been conducted to determine if 5 mg t.i.d. is the best dose of pilocarpine for Japanese who probably have a different body mass index than Westerners. The purpose of this study was to evaluate the tolerability of Japanese to 5 mg oral pilocarpine t.i.d. daily during or after radiotherapy in head and neck cancer.

2. Materials and methods

2.1. Patients

Thirty-nine patients, who had been suffering from radiation-induced xerostomia, received pilocarpine during or after radiotherapy in our institute. They started to receive pilocarpine between January 2006 and July 2006. All of the patients had head and neck carcinomas and received >50 Gy radiotherapy that included the parotid glands in the radiation field.

The patients' characteristics are summarized in Table 1. Thirty-nine patients consisted of 35 males and 4 females with a median age 61 years. The body weights of the patients ranged from 42 to 73 kg (median 60 kg). Twenty-eight (72%) patients had pharyngeal cancer. Five (13%) patients had laryngeal cancer, three (8%) had oral cancer, two (5%) had unknown primary cancer, and one (3%) had paranasal cavity cancer.

Twenty-three (59%) patients received definitive radiotherapy, while 16 (41%) patients received post-operative radiotherapy. Twenty (51%) patients received concurrent chemotherapy. The prescribed irradiation dose ranged from 60 to 72 Gy (median 70 Gy). The mean dose to both salivary glands ranged from 19.9 to 57.3 Gy (median 41.1 Gy).

Table 1
Patients' characteristics

Characteristics	$n = 39$
Age, years (median)	39–84 (61)
Gender (%)	
Female	10
Male	90
Body weights (%)	
<65 kg	67
>65 kg or =65 kg	33
Tumor site (%)	
Pharynx	72
Epipharynx	15
Mesopharynx	15
Hypopharynx	31
Larynx	13
Oral cavity	8
Primary unknown	5
Paranasal cavity	3
Intent of radiotherapy (%)	
Definitive radiotherapy	59
Post-operative radiotherapy	41
Prescription dose, Gy (median)	60–72 (70)
Mean dose of both salivary glands, Gy (median)	19.9–57.3 (41.1)
Concurrent chemotherapy (%)	
Yes	51
No	49
Duration from the beginning of radiotherapy to the beginning of pilocarpine (%)	
<120 or =120 days	15
>120 days	85

n = number of patients.

2.2. Pilocarpine

All patients received a daily dose of pilocarpine of 5 mg t.i.d. They were seen prior to initiation of treatment and at 2-week intervals thereafter, and continued to receive pilocarpine for at least 12 weeks whether effective or not unless they had experienced unacceptable adverse effects.

The duration from the beginning of radiotherapy to the start of pilocarpine ranged from 2 weeks to 72 months (median 8 months). Thirty-three (85%) patients started pilocarpine 4 months or later from the start of radiotherapy.

2.3. Study outcomes

The primary outcomes were determined by the rate that pilocarpine was maintained for 12 weeks, with or without any adverse effects (defined as tolerated rate). Secondary outcomes included the incidence of adverse effects, and the subjective symptoms of xerostomia.

Adverse effects were reported by telephone as they occurred, or at the bi-weekly appointments throughout the study.

The subjective assessment of efficacy was undertaken through the use of visual analog scales (VAS) every 4 weeks. The 100 mm visual analog scale (VAS) was used to record the response. Patients were asked to rate their condition of the dryness of the mouth on a scale from 0 to 100. This questionnaire was completed before starting pilocarpine for a period of 12 weeks. A patient with an increase of at least 25 mm from the baseline in the VAS score was defined as a "Responder." Response rate was defined as the total number of "Responder" divided by the number of maintaining patients. Response rate, in addition, was calculated among the 30 patients who started pilocarpine after more than 4 months from the start of radiotherapy and were considered to have fixed symptoms of xerostomia.

3. Results

Three of the 39 patients were excluded from the analysis because they stopped taking pilocarpine within 12 weeks for

Table 2
Tolerability of pilocarpine with 5 mg t.i.d.

Status	n	%
Tolerable without any adverse effects	6	17
Tolerable with some adverse effects	11	30
Unacceptable adverse effects	19	53

n = number of patients.

reasons other than adverse effects. (Two of them refused to continue because of insufficient efficacy, and one stopped because of beginning other medication which should not be used concurrently with pilocarpine.).

3.1. Tolerability

Of the remaining 36 patients, the tolerated rate was as low as 47%. Only 17 of the 36 patients were able to continue pilocarpine for 12 weeks with or without any adverse effects. Nineteen (53%) stopped taking pilocarpine within 12 weeks because of unacceptable adverse effects (Table 2).

The duration from the beginning to stopping pilocarpine due to adverse effects ranged from 3 to 42 days (median 7 days).

When we divide patients in less or more than 65 kg, the tolerated rates between two groups showed a significantly difference. Of the 25 patients whose body weights were less than 65 kg, the tolerated rate was 36%, whereas the tolerated rate of the 11 patients whose body weights were 65 kg or more was 72% ($p = 0.050$, calculated by a χ^2-test) (Table 3).

3.2. Incidence of adverse effects

The most common adverse effect was sweating, and its incidence was 64%. Other adverse effects reported included nausea, rhinitis, headache, cervical pain, fatigue, dazzling, oversalivation, and paresthesia of the tongue (Table 4).

Table 3
Tolerated rate according to the patients' body weights

Body weights	n	Tolerated rate (%)
<65 kg	25	36
>65 kg or =65 kg	11	72

n = number of patients; $p = 0.050$.

Table 4
Incidence of adverse effects with a probable relationship to pilocarpine

Adverse effects	% (n = 36)
Sweating	64
Nausea	8
Rhinitis	6
Headache	3
Cervical pain	3
Fatigue	3
Dazzling	3
Oversalivation	3

n = number of patients.

Table 5
Response rate

	Pretreatment	4 weeks	8 weeks	12 weeks
n	30	20	15	15
Response rate (%)	–	10	27	40

n = number of patients.

With the exception of paresthesia of the tongue, all of the other adverse effects which caused patients to quit taking pilocarpine disappeared within 1 week of stopping and were probably related to pilocarpine.

3.3. Subjective symptoms of xerostomia

Response rates at 4, 8, and 12 weeks were 10, 27, and 40%, respectively (Table 5).

4. Discussion

The most common adverse effect of pilocarpine is sweating, and its incidence is thought to be dose related. In the review of two prospective randomized trials that included 369 patients, Rieke et al. reported that the incidence of sweating with pilocarpine was 29% with 5 mg t.i.d, while it was 68% with 10 mg t.i.d. [11] They concluded that the adverse effects were considered acceptable by patients taking 5 mg t.i.d. They also reported that an improvement in dryness was obtained in 51% of patients receiving 5 mg t.i.d. pilocarpine, which was equally effective as 10 mg t.i.d, but 2.5 mg t.i.d was judged to be an ineffective dose.

Our investigation indicated that the incidence of sweating was 62%, and the tolerability was very low in spite of using 5 mg t.i.d. Conceivably, the physical difference between the Japanese and Westerners may explain the discrepancy between our results and those reported by Rieke et al. [11].

In our study, the tolerated rate in patients whose body weights were less than 65 kg was much lower than that in patients whose weights were 65 kg or above ($p = 0.050$). For Japanese patients, especially for those weighting 65 kg or less, 5 mg t.i.d. of pilocarpine appears to be an over dose. The proper dose of pilocarpine may be a little lower than 5 mg t.i.d. for the average Japanese, and perhaps the tolerability can be raised without decreasing efficacy when using a proper dose.

In conclusion, an oral pilocarpine dose of 5 mg t.i.d. caused a high incidence of unacceptable adverse effects for Japanese. A lower dose of pilocarpine needs to be considered in conjunction with body weights to find a proper dose.

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Umami taste dysfunction in patients receiving radiotherapy for head and neck cancer

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Summary Taste loss is a major cause of morbidity in patients undergoing head and neck irradiation. Previous studies have reported the alteration of the four basic tastes in patients with head and neck cancer during radiotherapy. However, only a few studies have been conducted on the effects of irradiation on the function of *umami* taste, a novel and basic taste recently recognized. In a prospective study, 52 patients undergoing radical head and neck irradiation were assessed for taste loss. Taste ability was measured by the taste threshold for *umami* quality using the whole-mouth taste method in patients before, during, and immediately after radiotherapy. *Umami* taste declined of the 3rd week after the start of radiotherapy and improved of the 8th week.

Introduction

Taste dysfunction is one of the most frequent complaints of patients undergoing radiation therapy (RT) for head and neck cancer. Complaints of taste disorders have been reported in 75% of patients with head and neck cancer undergoing radiation, and 93% of these patients complain of long-term xerostomia.[1] Many patients undergoing dose-intensive radiation experience reduced taste (ageusia) or altered taste (dysgeusia), which may have a significant impact on quality of life (QOL). Patients with taste disturbance experienced greater weight loss than those who did not report a change in taste.[2] On the other hand, patients with taste loss had a worse outcome than those did not lose their sense of taste and were able to maintain their food intake and nutritional support.[3] To design a diet that maximizes

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