BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **236,** 194–198 (1997)
ARTICLE NO. RC976931

Interleukin-1β-Converting Enzyme and CPP32 Are Involved in Ultraviolet B-Induced Apoptosis of SV40-Transformed Human Keratinocytes

Hidetoshi Takahashi, Motoshi Kinouchi, and Hajime Iizuka
Department of Dermatology, Asahikawa Medical College, Asahikawa 078, Japan

Received June 9, 1997

Interleukin-1β-converting enzyme (ICE) and CPP32 are cysteine proteinases, that are involved in apoptotic process in various cell systems. We investigated the effects of ICE on ultraviolet B (UVB) induced-apoptosis in SV40-transformed human keratinocytes (SVHK cells). The ICE inhibitor (Z-Val-Ala-Asp-CH_2F) and CPP32 inhibitor (Z-Asp-Glu-Val-Asp-CH_2F) blocked the apoptotic cell death caused by UVB irradiation. The addition of both ICE and CPP32 inhibitors to the incubation medium resulted in neither an additive nor a synergistic suppression of UVB-induced apoptosis. Reverse transcription and polymerase chain reaction (RT-PCR) analysis indicated that SVHK cells expressed ICE-α, and β mRNAs. UVB irradiation increased the mRNA of both isoforms and Western blot analysis confirmed that UVB increased an active form of ICE protein, p20, that is generated by autoproteolytic cleavage of inactive 45 kDa proenzyme derived from both mRNAs. Transfection of ICE expression vector into SVHK cells resulted in apoptosis in a dose dependent manner and UVB-irradiation further augmented the ICE expression vector-induced apoptosis. These results indicate that ICE plays an important role in UVB-induced apoptosis of SVHK cells.

Apoptosis, or programmed cell death, is a distinct mode of cell death that plays a crucial role in embryogenesis, carcinogenesis, and development of the immune system (1, 2). Cells undergoing apoptosis are morphologically characterized by a reduction of cell volume, membrane blebbing, and nuclear fragmentation (3). Wyllie demonstrated the presence of multiple 180 bp oligonucleosomal DNA fragments produced by activation of an endogenous endonuclease in apoptotic cells (4).

UVB (290-320 nm) irradiation produces "sunburn cells" in the epidermis (5, 6). The "sunburn cells" are keratinocytes that show eosinophilic cytoplasm with or without remnants of shrunken and condensed nuclei in hematoxylin and eosin staining (6, 7). "Sunburn cells" result from a specific type of apoptosis triggered by UVB-induced DNA damage.

Interleukin-1β converting enzyme (ICE) is a cysteine proteinase that cleaves inactive 31-kDa pro-IL-1β to generate active 17.5 kDa IL-1β (8). ICE is a 45 kDa protein and active ICE is produced by autoproteolytic cleavage generating two subunits: 20 kDa and 10 kDa proteins (8). Crystal structure analysis indicated that the catalytically active form of the enzyme is a tetramer consisting of (p20-p10)2 homodimers (8). Recent studies suggest that in addition to IL-1β activation, ICE or ICE-like cysteine proteinase plays a role in the induction of apoptosis. CPP32 is another cysteine proteinase and active CPP32, which consists of p17 and p12, is cleaved by prodomain of the ICE/CPP32 family. The CPP32 mRNA is detected in various cells including squamous cell lines, such as A431 and HeLa cells (9). The inhibitors of ICE and CPP32 block the apoptosis induced by Fas antigen, tumor necrosis factor-α (TNF-α), and anticancer agents in various cell systems (10-12). However, it is unclear whether these proteinases participate in a common signaling pathway of apoptosis.

SV40-transformed human keratinocytes (SVHK cells) are a well-established immortalized cell line sharing features of normal human keratinocytes (13). In the present study we used SVHK cells to investigate the role of ICE and CPP32 in the UVB-induced apoptosis.

Abbreviations: ICE, interleukin-1b-converting enzyme; IL-1, interleukin-1; PBS, phosphate buffered saline; RT-PCR, reverse transcription and polymerase chain reaction; SVHK, SV40-transformed human keratinocytes; TNF-α, tumor necrosis factor-α; UVB, ultraviolet B.

MATERIALS AND METHODS

Cell culture. SVHK cells (13) were a generous gift from Dr. M. L. Steinberg (Department of Chemistry, City College of the City Univer-

0006-291X/97 $25.00

sity of New York). Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin at 37 °C in 5% CO_2 in air.

Cell death assay. SVHK cells were seeded at 1×10^4 cells in a 96-well microtiter plate and pre-incubated with ICE inhibitor (Z-Val-Ala-Asp-CH_2F) or CPP32 inhibitor (Z-Asp-Glu-Val-Asp-CH_2F) for 2 hr. Prior to UVB irradiation, the cultured cells were washed twice with phosphate-buffered saline (PBS) (pH 7.5) to remove the photosensitizer, phenol red, present in the Eagle's medium. The UVB irradiation source was a Toshiba-Eizai Dermaray instrument (DMR-1, Tokyo, Japan) with 5 fluorescent lamps (FL-20-SE-30, Toshiba, Japan). After irradiation, the cells were incubated again with various concentrations of ICE inhibitor or CPP32 inhibitor. Cell death assay was performed according to the method of Itoh (14). The cells were stained for 20 min at room temperature with 0.75% crystal violet in 50% ethanol, 0.25% NaCl, and 1.75% formaldehyde, and were washed three times with PBS. During the washing procedure, dead cells were removed leaving viable cells in the well. Dye uptake was quantified by measuring OD at 540 nm using an automated Micro-ELISA autoreader. The ratio of viable cells was expressed as a percentage of the OD value obtained without UVB treatment.

Analyses of DNA fragmentation. 1×10^6 cells were suspended in 100ml of 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100. After incubation at 4 °C for 20 min., the cell lysate was centrifuged and the supernatant was incubated in the presence of 0.4 mg/ml RNase A at 37°C for 1h. Then 2ml proteinase K (20 mg/ml) was added to the reaction mixture and incubation was continued at 37 °C for 1h. Samples were electrophoresed on 2% agarose gels.

Cloning of ICE isoforms. The cDNA of ICE isoforms were cloned by reverse transcription and polymerase chain reaction techniques (RT-PCR) according to the method of Alnemri et al. (15). Briefly, reverse transcription was performed on total RNA (5 μg) from SVHK cells using an ICE-RT primer (5′-CAGAACGATCTCTTCAC-3′) and Molony murine leukemia virus reverse transcriptase. The reverse transcription products were used as the template for PCR using ICE1 (5′-ATGGCCGACAAGGTCCTG-3′) and ICE2 (5′-CCTGCCCGC-AGACATTCA-3′) primers. The amplification of β-actin by RT-PCR was performed as previously described (16). The PCR products were subcloned to TA cloning vector, pCRTM II (pCR-ICE), and confirmed by DNA sequence analysis.

Western blotting. Cytosolic protein was extracted with cell lysis buffer (50 mM Tris-HCl, pH 7.4; 1% NP-40; 0.25% sodium deoxycholate; 150 mM NaCl; 1 mM EGTA; 1 mM phenylmethylsulfonyl fluoride; 1 mg/ml aprotinin, leupeptin, pepstatin; 1 mM sodium vanadate; 1 mM NaF) and electrophoresed on a 12.5% SDS-PAGE gel and electroblotted onto nitrocellulose for 1 hr. in a buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol. The blots were blocked with 5% nonfat milk in PBS for 1 hr. at room temperature, and then incubated at 4 °C overnight with anti-human ICE antibody (Upstate Biotechnology, Lake Placid, NY) that had been diluted 1000-fold in Tris-buffered saline (TBS, pH 7.6). After washing at room temperature with 0.1% Tween-20 in TBS, a blotting detection kit for rabbit antibody (Amersham International plc, UK) was used for immunodetection.

Construction of plasmid and transfection. ICE cDNA was digested by restriction enzyme, EcoR I from pCR-ICE vector and ligated to the EcoR I sites of pSG5 vector (pSG-ICE). 4 μg of pSG5-ICE vector was transfected into 1×10^5 SVHK cells using lipofectin. After 48 hr., cells were collected and a cell death assay was performed.

Materials. Dulbecco's modified Eagle's medium was purchased from Gibco (Grand Island, NY). Penicillin and streptomycin were obtained from M.A. Bioproducts (Walkersville, MD). Lipofectin and Molony murine leukemia virus reverse transcriptase were obtained from BRL (Bethesda, MD). DNase and RNase inhibitor (RNasin) were purchased from Promega (Madison, WI). Taq polymerase was purchased from Perkin Elmer-Cetus (Norwalk, CT). ICE inhibitor (Z-Val-Ala-Asp-CH_2F) and CPP32 inhibitor (Z-Asp-Glu-Val-Asp-CH_2F) were purchased from Kamiya Biomedical Company (Seattle, WA). pSG5 expression vector was purchased from Stratagene (La Jolla, CA). TA cloning vector, pCRTM II was purchased from Invitrogen Corporation (San Diego CA). All other chemicals were obtained from Nakarai Chemicals Ltd. (Kyoto, Japan).

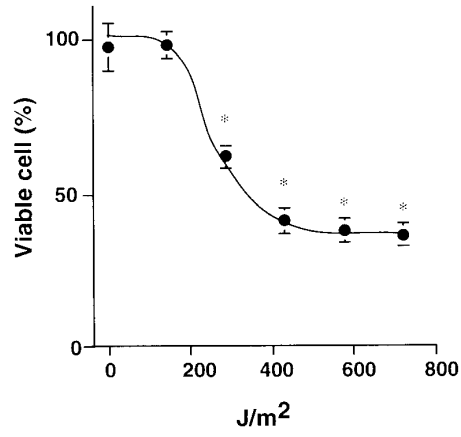

FIG. 1. Induction of apoptosis by UVB irradiation in SVHK cells. Following UVB irradiation at various UVB doses, SVHK cells were cultured for 24 hr and viable cells were measured. Dye uptake was quantitated by measuring the OD at 540 nm. Data are expressed as percentage of viable cells±S.E. (all n=4). *P<0.01 compared to control.

RESULTS

UVB irradiation-induced apoptosis of SVHK cells. The maximal induction of apoptosis was observed at 600 J/m (Fig. 1). As shown in Fig. 2, lane 2, 600 J/m^2 UVB irradiation induced a typical internucleosomal DNA fragmentation. No DNA fragmentation was detected in unstimulated SVHK cells (Fig. 2, lane 1). UVB-induced apoptosis was time-dependent and was detected by 3 hr., with the maximal effect observed by 24 hr. (Fig. 3A).

ICE and CPP32 inhibitors prevent UVB irradiation-induced apoptosis. UVB-induced apoptosis was suppressed by ICE inhibitor (Z-Val-Ala-Asp-CH_2F). The effect of ICE inhibitor was detected by 6 hr. and remained for at least 24 hr. (Fig. 3A). The suppressive effect of ICE inhibitor was concentration-dependent (Fig. 3B, lane 4); and was detected at concentrations as low as 1 μM and was most marked at 10 μM.

UVB-induced apoptosis was also suppressed by CPP32 inhibitor (Z-Asp-Glu-Val-Asp-CH_2F) (Fig. 4). The suppressive effects of the two inhibitors on apoptosis were almost the same at the concentration used (10μM). The addition of both ICE and CPP32 inhibitors to the incubation medium slightly augmented

the suppression of UVB-induced apoptosis. This was, however, statistically not significant.

UVB irradiation increases the expression of ICE-α and -β in SVHK cells. ICE mRNA is composed of at least five isoforms (α, β, γ, δ, and ε) that are derived from a single gene by alternative splicing (16). RT-PCR analyses indicated that SVHK cells constitutively express ICE-α (1248bp), and -β (1185bp) mRNAs (Fig. 5, lane 1). UVB irradiation increased the expression of mRNA of both ICE isoforms, which was detected by 3 hr. and returned to the basal level by 24 hr. (Fig. 5). Quantitative analysis indicated that the induction was three-fold (3 hr.) and four-fold (6 hr.), respectively, compared to the peak control values. The expression of β-actin mRNA was not altered by the UVB irradiation. Other ICE isoforms mRNAs (γ, δ, ε) were not induced by UVB irradiation (data not shown). Western blot analysis also revealed that the active form of ICE protein, p20, was increased following UVB irradiation in SVHK cells. The polyclonal antibody reacted slightly with the 22kDa protein. The effect was observed by 3 hr. and the maximal effect was detected by 48 hr. (Fig. 6). The p20 expression was marginally detectable in unstimulated SVHK cells (Fig. 6, lane 1).

UVB irradiation-induced apoptosis is augmented by the transfection of ICE expression vector. To examine the direct effect of ICE proteinase in inducing apoptosis, we transfected ICE-α expression vector, pSG5-ICE, into SVHK cells. Transfection of pSG5-ICE vector induced apoptosis in SVHK cells, that was accompanied by fragmentation of DNA (Fig. 2, lane 3).

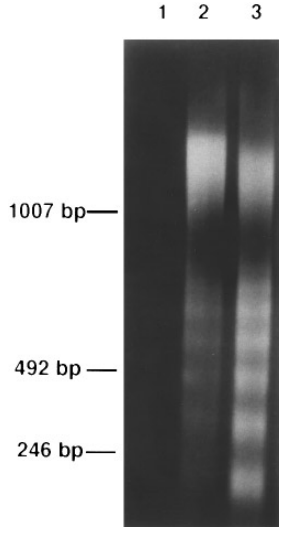

FIG. 2. DNA fragmentation induced by UVB irradiation or by transfection of pSG5-ICE vector in SVHK cells. SVHK cells were irradiated by UVB (600 J/m^2), or transfected by pSG5-ICE-α vector (4 μg), and then cultured for 24 hr. or 48 hr., respectively. 1: control, 2: UVB treatment, 3: transfection of pSG5-ICE-α vector.

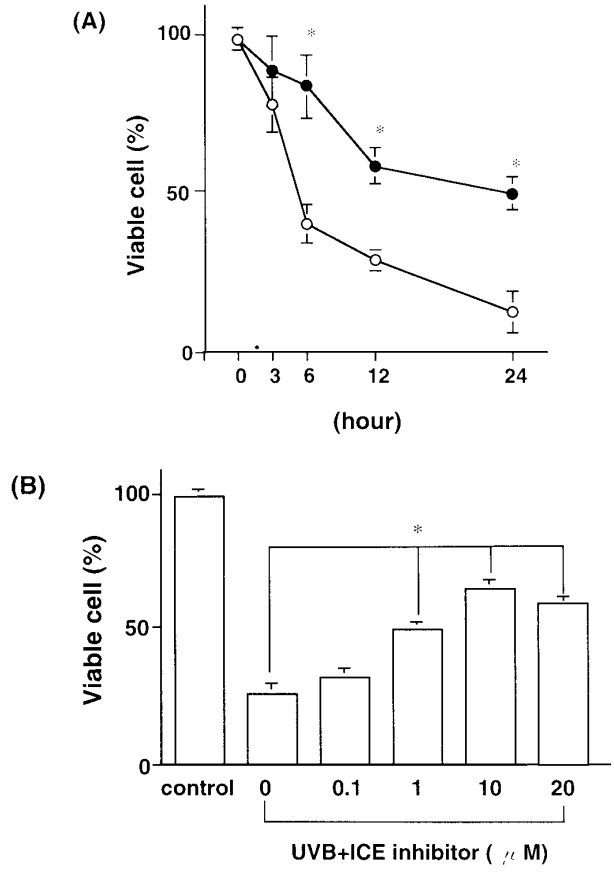

FIG. 3. Effects of ICE inhibitor on UVB-induced apoptosis in SVHK cells. (A) Following pre-treatment with or without ICE inhibitor (Z-Val-Ala-Asp-CH_2F) (10 μM), SVHK cells were irradiated by 600J/m^2 UVB, and cultured for the indicated time. Viable cells were evaluated by measuring the OD at 540 nm. Open circle is in the absence of ICE inhibitor. Closed circle is in the presence of ICE inhibitor. *P<0.01 compared to control. (B) Following pre-treatment with or without ICE inhibitor, SVHK cells were irradiated by 600J/m^2 UVB, and cultured for 24 hr. with various concentrations of ICE inhibitor. Following the culture, the viable cells were measured. 1:medium, 2:UVB irradiation, 3:UVB irradiation in the presence of ICE inhibitor (0.1 μM), 4:UVB irradiation in the presence of ICE inhibitor (1 μM), 5:UVB irradiation in the presence of ICE inhibitor (10 μM), 6:UVB irradiation in the presence of ICE inhibitor (20 μM). Data are expressed as percentage of viable cells±S.E. (all n=4). *P<0.01 compared to UVB-irradiated SVHK cells.

The effect of ICE expression vector was concentration-dependent (Fig. 7). In addition, UVB-induced apoptosis was further augmented by pSG5-ICE in SVHK cells (Fig. 7, lane 7). Transfection of ICE-β expression vector also induced apoptosis to a similar extent as that of ICE-α (data not shown).

DISCUSSION

ICE cysteine proteinase is a mammalian homologue of CED-3, a positive regulator of apoptosis in the nema-

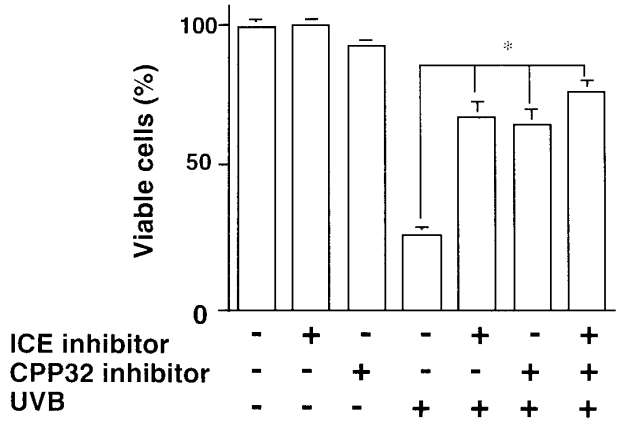

FIG. 4. Effects of inhibitors of ICE and CPP32 on UVB-induced apoptosis. Following pre-treatment with or without ICE inhibitor or CPP32 inhibitor, SVHK cells were irradiated by 600J/m2 UVB, and cultured with ICE inhibitor (Z-Val-Ala-Asp-CH_2F) (10 μM), CPP32 inhibitor (Z-Asp-Glu-Val-Asp-CH_2F) (10 μM), or both for 24 hr. Following the culture, the viable cells were measured. Data are expressed as percentage of viable cells±S.E. (all n=4). *P<0.01 compared to UVB-irradiated SVHK cells.

tode Caenorhabditis elegans (17). It has been reported that apoptosis, which is induced by Fas ligand, TNF-α, or antitumor agents, is blocked by the treatment of ICE family proteinase inhibitors (10-12). Our results indicate that ICE inhibitor (Z-Val-Ala-Asp-CH_2F) also blocks UVB-induced apoptosis, suggesting that ICE plays an important role in the induction of UVB induced-apoptosis in SVHK cells. CPP32/Yama/apopain proteinase is another cysteine proteinase that affects the apoptotic process (9, 18). Using a pheochromocytoma cell line, Shimizu et al. reported that CPP32/Yama/apopain(-like) proteinase was activated following ICE(-like) proteinase activation (19). In our study CPP32 inhibitor (Z-Asp-Glu-Val-Asp-CH_2F) also blocked the UVB-induced apoptosis and the addition of both ICE and CPP32 inhibitors did not augment the

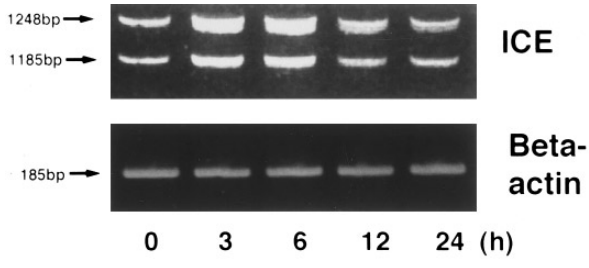

FIG. 5. Induction of ICE mRNA by UVB irradiation in SVHK cells. SVHK cells were irradiated by UVB (600 J/m2), and were cultured for the indicated time. RT-PCR was performed using 5μg total RNA. The products of 1248bp and 1185bp indicate the ICE-α and β isoforms, respectively. 185pb is the PCR product from β-actin as the control.

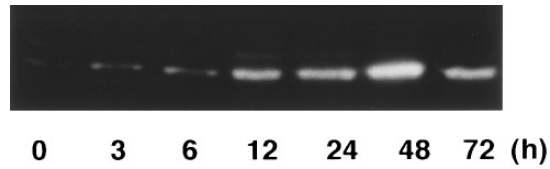

FIG. 6. Induction of active p20 ICE protein by UVB irradiation in SVHK cells. SVHK cells were irradiated by UVB (600 J/m2), and were cultured for the indicated time. Cell lysates were extracted and Western blot analysis was performed.

suppressive effect. These results suggest that ICE and CPP32 proteinase are located on the same cascade of the apoptotic process following UVB irradiation.

ICE mRNAs consist of at least five isoforms derived from alternative splicing of a single gene (9). SVHK cells have ICE-α and -β mRNA isoforms. ICE-α mRNA is the full length form, while ICE-β mRNA is 63 bp shorter due to splicing out of the 2nd exon. Our results indicate that transfection of ICE-α or -β expression vector into SVHK cells induces apoptosis. UVB irradiation augmented the induction of apoptosis in ICE expression vector-transfected SVHK cells. This again is consistent with the idea that ICE proteinase plays a role in the induction of apoptosis.

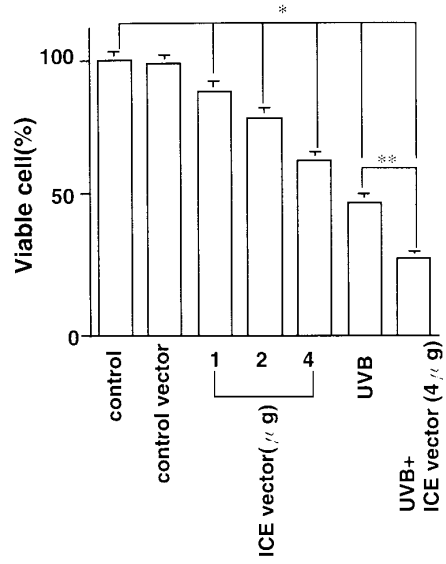

FIG. 7. Induction of apoptosis by the transfection of pSG5-ICE-α vector into SVHK cells. Various concentrations of pSG5-ICE vector were transfected into SVHK cells, and were cultured for 48h. UVB irradiation (600J/m^2) was performed as in Fig. 3. Data are expressed as percentage of viable cells±S.E. (all n=4). *P<0.01 compared to control. **P<0.01 compared to UVB irradiation (lane 6). lane 1: control, lane 2:transfection of pSG5 control vector (4 μg), lane 3: transfection of pSG5-ICE-α vector (1μg), lane 4:transfection of pSG5-ICE-α vector (2μg), lane 5: transfection of pSG5-ICE-α (4μg), lane 6:UVB irradiation (600J/m^2), lane 7: transfection of pSG5-ICE-α (4 μg) and UVB irradiation (600 J/m^2).

The active form of ICE is derived from a precursor protein, p45, by proteolytic cleavage at Asp103, Asp119, Asp297, and Asp316 (20). The active ICE consists of p20 (residues 120-297) and p10 (residues 317-404), both of which are essential for the enzymatic activity. Western blot analysis showed that the increased expression of p20 correlated with the UVB-dependent induction of apoptosis. The polyclonal antibody against human ICE protein recognizes only the p20 subunit under reducing conditions, which we performed. Although the antibody strongly reacted with the 20kDa protein, it also reacted slightly with the 22kDa protein. The nature of the 22kDa protein remains unknown.

RT-PCR analysis showed that ICE (α and β) mRNAs are constitutively expressed in SVHK cells. Still they were markedly induced by UVB irradiation. The time course of the increased mRNA correlated well with the induction of apoptotic cells by UVB irradiation. Miura et al. reported that overexpression of the ICE gene induced apoptosis in a rat fibroblast cell line (17). These results also offer an explanation for the augmentation of apoptotic cells by UVB-irradiation in ICE transfected SVHK cells.

The epidermis is one of the target tissues of apoptosis. Apoptosis is observed not only following the UVB irradiation but also in the hair follicles during the catagen phase of the hair cycle, lichenoid skin disorders, and tumor regression (2). In addition, activation of Fas antigen also induces apoptosis. Fas antigen is highly expressed in psoriasis, lichen planus, bullous pemphigoid, and erythema multiforme (21). Previously we showed that anti-Fas antibody induces the apoptosis in interferon-g-treated SVHK cells (22). Enari et al. reported that ICE-like and CPP32-like activities were increased following the Fas antigen stimulation in a mouse transformant lymphoma cell line, W4, which constitutively expresses mouse Fas antigen (10). Recent evidence indicates that Fas-dependent apoptosis is also blocked by ICE or CPP32 inhibitors (10). These findings suggest the possibility of systemic or topical application of ICE or CPP32 inhibitors as treatment for psoriasis, lichen planus, bullous pemphigoid, and erythema multiforme.

ACKNOWLEDGMENTS

This study was supported in part by Grants 08457233 (H.I.) and 08770618 (H.T.) from the Ministry of Education, Science, Sports and Culture of Japan and from the Ministry of Health and Welfare, Japan. The technical assistance of Mrs. Y. Kotani and Mrs. K. Takahashi and the secretarial assistance of Miss Y. Maekawa are greatly appreciated.

REFERENCES

1. Williams, G. T. (1991) *Cell* **65,** 1097–1098.
2. Haake, A. R., and Polakowska, R. R. (1993) *J. Invest. Dermatol.* **101,** 107–112.
3. Kerr, J. F. R., Wyllie, A. H., and Currie, A. R. (1980) *Brit. J. Cancer* **26,** 239–257.
4. Wyllie, A. H. (1980) *Nature* **284,** 555–556.
5. Olson, R. L., Gaylor, J., and Everett, M. A. (1974) *J. Cutan. Pathol.* **1,** 120–125.
6. Iizuka, H., Ishida-Yamamoto, A., Kajita, S., Tsutsui, M., and Ohkuma, N. (1979) *Arch. Dermatol. Res.* **280,** 163–167.
7. Weedon, D., Seale, J., and Kerr, J. F. R. (1979) *Am. J. Dermatopathol.* **1,** 133–144.
8. Kumar, S. (1995) *TIBS* **20,** 198–202.
9. Fernandes-Alnemri, T., Litwack, G., and Alnermi, E. S. (1994) *J. Biol. Chem.* **269,** 30761–30764.
10. Enari, M., Talanian, R. V., Wong, W. W., and Nagata, S. (1996) *Nature* **380,** 723–726.
11. Gagliardini, V., Fernandez, P. A., Lee, R. K., Drexler, H. C., Rotello, R. J., Fishman, M. C., and Yuan, J. (1994) *Science* **263,** 826–828.
12. Mashima, T., Naito, M., Kataoka, S., Kawai, H., and Tsuruo, T. (1995) *Biochem. Biophys. Res. Commun.* **209,** 907–915.
13. Takahashi, H., Tamura, T., Tsutsui, M., and Iizuka, H. (1990) *J. Dermatol. (Tokyo)* **17,** 457–464.
14. Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991) *Cell* **66,** 233–243.
15. Alnemri, E. S., Fernandes-Alnemri, T., and Litwack, G. (1995) *J. Biol. Chem.* **270,** 4323–4317.
16. Takahashi, H., Kinouchi, M., Tamura, T., and Iizuka, H. (1996) *Br. J. Dermatol.* **134,** 1065–1069.
17. Miura, M., Zhu, H., Rotello, R., Hartwieg, E. A., and Yuan, J. (1993) *Cell* **75,** 653–60.
18. Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995) *Cell* **81,** 801–809.
19. Shimizu, S., Eguchi, Y., Kamiike, W., Matsuda, H., and Tsujimoto, Y. (1996) *Oncogene* **12,** 2251–2257.
20. Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., and Aunins, J. (1992) *Nature* **356,** 768–74.
21. Sayama, K., Yonehara, S., Watanabe, Y., and Miki, Y. (1994) *J. Invest. Dermatol.* **103,** 330–334.
22. Takahashi, H., Kobayashi, H., Hashimoto, Y., Matsuo, S., and Iizuka, H. (1995) *J. Invest. Dermatol.* **105,** 810–815.