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Increased Ecto-Metallopeptidase Activity in Cells Undergoing Apoptosis

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Abstract Release from the cell surface of a variety of growth factors, cytokines, and proteases follows exposure to genetically stressful agents capable of inducing apoptosis and necrosis. Increased ectoprotease activity is responsible for their release. We show that increased activity of several metalloproteases on the HeLa cell surface occurs after stresses due to UVC, actinomycin D, cycloheximide, and cisplatinum, which induce the release of transforming growth factor-α (TGFα) and other bioactive molecules. The ectoprotease activities increase preferentially on apoptotic cells, while little change occurs in viable cells. Gross decreases, except for the putative TGFαase activity, accompany necrosis. These changes may contribute to tissue repair and the absence of an inflammatory reaction to apoptotic cell death. They appear to be due to preferential enzyme activation or to retention by cells undergoing significant categorical decreases in protein content. J. Cell. Biochem. 76:625–638, 2000.

Key words: apoptosis; necrosis; HeLa cells; metallopeptidase; aminopeptidase; TGFαase

Cells exposed to a lethal dose of a genotoxic agent die either by apoptosis or by necrosis. Kerr et al. [1972] were the first to name apoptosis; it has since been shown that this mode of cell death differs from that of necrosis with respect to many aspects of cell biology [Wyllie, 1997]. One of the early morphological changes that occur as an adherent cultured cell undergoes apoptosis is detachment from the vessel substrate, usually accompanied by the loss of specialized membrane structures such as microvilli [Kerr et al., 1987]. The cell then undergoes a period of rounding, shrinkage, and surface blebbing before condensation of chromatin is observed in the nucleus. Biochemical changes that occur on or near the membrane during this period include the inversion of phosphatidylserine in the plasma membrane [Fadok et al., 1992; Savill et al., 1993], activation of a cascade of caspases [Cohen, 1997], and the cleavage of an increasing list of proteins including Gelsolin, PAK2 and α-fodrin [Cryns and Yuan, 1998; Porter and Jänicke, 1999]. These caspases have been shown to be intracellular and are directly involved in the intracellular events that occur as the cell undergoes apoptosis. Other proteases apart from the caspases have been shown to be activated during apoptosis, including calpains [Squier and Cohen, 1996] and serine proteases [Sukharev et al., 1997].

The roles played by metalloproteases in apoptosis have not been examined in any significant detail. Members of this family of proteases have been shown to cleave growth factors [Arribas et al., 1996; Piva et al., 1997; Sukharev et al., 1997], cytokines [Black et al., 1997] and various immunomodulatory molecules [Hooper et al., 1997; Tanaka et al., 1998] from precursors resident in the cell membrane. Recent studies have shown that metalloproteases were responsible for the shedding of the IgG receptor FcγRIIIb (CD16) from neutrophils [Middelhoven et al., 1997] and for upregulation of an ectopeptidase

Abbreviations used: ActD, actinomycin D; Ac-YVAD-CHO, Ac-Tyr-Val-Ala-Asp-CHO; Ac-YVAD-pNA, Ac-Tyr-Val-Ala-Asp-p-nitroanilide; Cisplatin, cisplatinum; CHX, cycloheximide; eAP, ecto-aminopeptidase; eDP, ecto-dipeptidase; eTP, ecto-tripeptidase; FBS, fetal bovine serum; H33342, Hoechst 33342; ICE, interleukin-1β-converting enzyme; PI, propidium iodide.

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with the cleavage specificity expected of a TGFαase [Piva et al., 1997] and aminopeptidase activity in HeLa cells [Piva et al., 1998] undergoing apoptosis.

After exposure to ultraviolet (UV) irradiation, some skin keratinocytes undergo apoptosis and are then called "sunburn cells" [Danno and Horio, 1987]. Keratinocytes after exposure to UV irradiation have been shown to release a range of molecules from the cell surface including TGFα [Murphy et al., 1991], TNFα [Schwarz et al., 1995] and Fas ligand [Gutierrez-Steil et al., 1998]. The enzymes responsible for the cleavage of these molecules are metalloproteases, but it is unknown whether elevation of their activities is responsible for the shedding of these surface markers or whether release occurs by other mechanisms. It also remains to be determined whether enzyme changes occur in all cells or just those that undergo apoptosis. TGFα levels have been shown to be elevated in human skin after exposure to UV [Murphy et al., 1991], and this may be attributable to increased release from melanocytes and keratinocytes [Ellem et al., 1988]. Previous studies using cultured HeLa cells have shown that UV irradiation increased the level of "TGFαase" and aminopeptidase activity, which reached maximal levels (2- to 5-fold) at 16–20 h postirradiation, when assayed with either intact cells [Brown et al., 1993] or purified cell membranes [Piva et al., 1997]. Preliminary studies have shown that there was an increase in the activity of other ecto-metallopeptidases in UV-irradiated HeLa cells undergoing apoptosis [Piva et al., 1998].

In this study, we have examined the effect of a variety of apoptosis-inducing agents on the activity of cell surface peptidases, in order to determine whether the increased activity observed in UV-irradiated apoptotic cells is part of a global response of all cells or is a manifestation of an early change in the process of cell death. We show that, with the exception of ecto-aminopeptidase, increased cell surface peptidase activity is observed only in apoptotic subpopulations of cells from cultures treated with genotoxic agents or that occur "spontaneously," in control cultures. Necrotic cells obtained from treated cultures, but not those from control cultures, exhibit reduced levels of cell surface peptidase activity. The results suggest that increased cell surface peptidase activity is not part of a global response to genotoxic agents, but it is an early change in the biochemical cascade that results in programmed cell death. This is the first study in which metalloproteases have been shown to undergo apoptotic activation.

MATERIALS AND METHODS

All chemicals and biochemicals were obtained from sources previously described [Piva et al., 1997], except for caspase-1 substrate N-acetyl-Tyr-Val-Ala-Asp-p-nitroanilide (Ac-YVAD-pNA) and inhibitor N-acetyl-Tyr-Val-Ala-Asp-CHO (Ac-YVAD-CHO) obtained from Biomol (Plymouth Meeting, PA); Pronase obtained from Calbiochem-Novabiochem (Bad Soden, Germany), and DNA molecular-weight standards VI obtained from Boehringer-Mannheim (Mannheim, Germany).

Cell Culture

HeLa S_3 cell monolayers were grown in RPMI 1640 containing penicillin (100 μg/ml), streptomycin (100 μg/ml), Hepes (20 mM), and 5% (v/v) fetal bovine serum (FBS) (pH 7.2 at 37°C), under sterile conditions. Trypsinized cells from 3–4 confluent 175-cm^2 tissue culture flasks were collected and washed with Phenol red free-Hepes-buffered saline solution (HBSS) before being suspended at 10^7 cells/ml Hank's Balanced Salt Solution (HBSS). In this study, 1-ml aliquots of cells (10^7) were transferred either to 60-mm tissue culture dishes (Iwaki Glass, Chiba, Japan) that were subsequently irradiated with UVC [Piva et al., 1997] or to 175-cm^2 flask containing 50 ml RPMI plus 2% FBS, as well as one of the after metabolic inhibitors: no inhibitor (control cells), cycloheximide (CHX) (10 μg/ml or 35.6 μM), actinomycin D (ActD) (1 μM), or cisplatinum (Cisplatin) (5 μg/ml or 16.7 μM). The cells were placed in a 37°C incubator for 20 h before being harvested for fluorescence-activated cell sorting (FACS) analysis.

Preparation of Cells for Sorting by Flow Cytometry

The detached cells present in tissue culture media of the treated cultures were transferred to 50-ml Falcon tubes. The attached cells were removed by trypsinization and placed in the same tubes. Cells were detached from the surface of the flasks using trypsin because (1) the percentage of viable cells recovered from the control flasks was greater than that obtained

by using a cell scraper (88.4% vs 54.1%, respectively), and (2) there was no difference in the level of cell surface protease activity between the two methods of cell detachment (data not shown). The cells were centrifuged (37°C at 200*g* for 5 min) and resuspended at 37°C in 5 ml RPMI containing 2% FBS. The cell populations were then stained with Hoechst 33342 (H33342) and propidium iodide (PI) before FACS analysis using the method described by Sun et al. [1992]. Briefly, H33342 (1 μg/ml final concentration) was added to the cells, which were returned to the 37°C incubator for 15 min, after which the cells were rapidly chilled by being placed on ice for 5 min before being centrifuged (4°C at 200*g* for 5 min). The cells were resuspended at 4°C in 1 ml RPMI containing 2% FBS to which PI (5 μg/ml final concentration) was added. The cells were passed through gauze into 5-ml Falcon tubes before being sorted on a Becton Dickinson FACS Vantage.

Flow Cytometry

The stained cells were separated into viable, apoptotic, and necrotic populations on the basis of their staining for PI and H33342 dyes as described previously [Piva et al., 1998]. The efficiency of sorting was examined by resorting the sorted subpopulations of HeLa cells on the FACS Vantage. The efficiency of sorting using the FACS Vantage was shown to be greater than 95%. The sorted cell populations were centrifuged (200*g* for 5 min at 4°C) before being washed twice with ice-cold HBSS. The sorted cell subpopulations were washed to remove any serum present in the collection medium, as FBS displays some protease activity [Piva et al., 1997]. After the final wash, the cells were suspended at 10^6 cells/ml HBSS and 50-μl aliquots placed in Eppendorf tubes. These tubes were placed in a 37°C incubator for 15 min to warm the cells before being assayed for cell surface protease activity.

Quantification of Apoptosis

DNA isolation and analysis. The DNA present in the sorted cell populations was isolated using the method of Piva [1994], with the following modification. The sorted cell populations were centrifuged (200*g* for 5 min at 4°C) before being washed with ice-cold HBSS. The cell pellet was resuspended in 0.5 ml HBSS and transferred to a centrifuge tube containing 2× Pronase buffer (100 mM NaCl, 10 mM EDTA, 20 mM Tris, 1% sodium dodecyl sulfate [SDS], pH 8.0) plus 0.5 mg Pronase. The tubes were left at room temperature overnight and the DNA extracted as described. 10 μg DNA was added to each lane of a 1.5% agarose gel that was run over 2–3 h at 60–80 mA. DNA standards (0.5 μg) were added to a lane in each gel. The gel was photographed under UV light (Polaroid Film 57).

Light microscopy. The sorted cell populations were centrifuged (200*g* for 5 min at 4°C) before being washed with ice-cold HBSS. The cell pellets were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, overnight at 4°C. They were then postfixed in 1% osmium tetroxide, sectioned, and stained with toluidine blue before examination under light microscopy, as described by Harmon et al. [1990].

Peptidase Activity

The P9 octapeptide (YVAAAVVSH), which is cognate to the N-terminal cleavage site of preproTGFα, was labeled with ^{125}I as previously described [Brown et al., 1992]. The rate of P9 hydrolysis by the sorted cell populations was assayed as follows: 20 μl of labeled P9 (1 μl ^{125}I-P9 (10 pmoles) in 20 μl HBSS) was added to a 1.5-ml Eppendorf tube along with 50 μl of FACS-sorted cell population (prewarmed to 37°C for 15 min) for 15 min (P9 final concentration 140 nM). The aminopeptidase inhibitor bestatin [Suda et al., 1976] was added to the assay to a final concentration of 10 μM. At the end of the experiment, 40 μl of the reaction mixture was spotted on a multichanneled thin-layer chromatography (TLC) plate. The plates were dried and the peptide fragments separated by ascending chromatography [butanol: H_2O:acetic acid, 100:30:10, pH 2.6], then imaged and analyzed by phosphorimaging analysis as previously described [Brown et al., 1992].

Cell Surface Protease Inhibition Studies

The protease inhibitors, including the interleukin-1β-converting enzyme (ICE) inhibitor Ac-YVAD-CHO [Nicholson and Thornberry, 1997], were added to the sorted cell populations for 15 min before the addition of ^{125}I-P9. The final concentrations of the added inhibitors were: Ac-YVAD-CHO (1 μM) and O-phenanthroline (0.5 mM).

ICE activity was measured using the method of Thornberry [1994]. Sorted cells were collected and washed twice with ice-cold HBSS before being resuspended in 100 mM Hepes, 10% sucrose, 0.1% CHAPS, and 10 mM DTT (pH 7.5). The reaction was commenced by the addition of 17.5 µM Ac-YVAD-pNA; the change in absorbance at 400 nm was recorded over 10 min. The rate of peptide cleavage was calculated through the use of Beer-Lambert's relationship, using 9160 $M^{-1}cm^{-1}$ as the extinction coefficient for Ac-YVAD-pNA [Thornberry, 1994]. The inhibitory effect of 1 µM Ac-YVAD-CHO on ICE activity was measured by adding it to the reaction mixture 15 min before the addition of substrate.

Calculations

The pmoles of P9-derived peptides separated by TLC were quantified using phosphorimage analysis. The rates of P9 hydrolysis were expressed as a function of cellular protein levels. The significance of differences attributable to apoptotic agents on the hydrolysis of P9 by HeLa cell subpopulations was determined using Student's *t*-test, and Bonferroni's adjustment of this analysis when necessitated by multiple comparisons.

RESULTS

The separation of viable, apoptotic, and necrotic cells from a population of HeLa cells was made by FACS analysis and sorting (Fig. 1: R_1, R_2,and R_3, respectively). The three populations were distinguished by their staining characteristics with PI and H33342 dyes similar to those seen in previous studies [Sun et al., 1992; Brown et al., 1996; Piva et al., 1997]. Microscopic examination showed typical purity of the three separate subpopulations as well as their morphological characteristics (Fig. 2): apoptotic cells were shown to have condensed chromatin and dense cytoplasm, as compared with viable and necrotic cells. The latter were distinguished by swollen organelles and ruptured membranes. Extracted DNA was electrophoresed (Fig. 3), and that from apoptotic cells displayed the characteristic ladder pattern [Wyllie, 1980], while DNA from viable cells remained as a high-molecular-weight zone near the origin and that from necrotic cells resulted in a typical smear pattern.

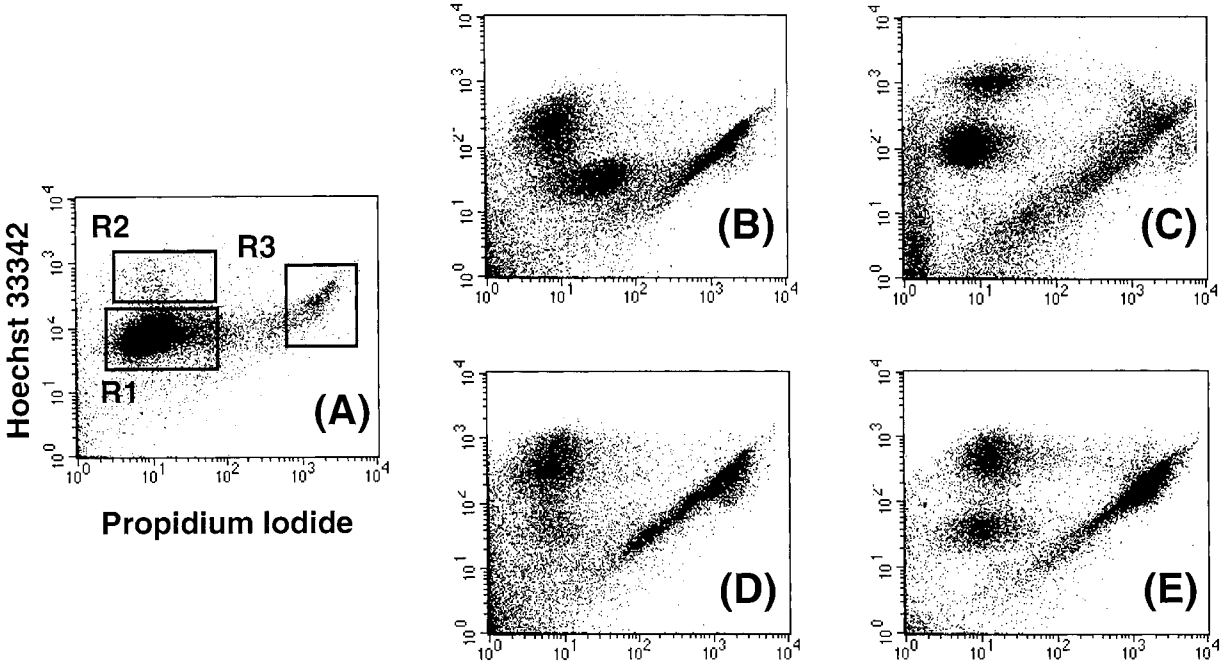

Fig. 1. Separation of HeLa cell subpopulations by flow cytometry. HeLa cells exposed to various apoptotic agents, collected from flasks 20 h after exposure and incubated with H33342 and PI as described under Materials and Methods. The cells were separated by flow cytometry. Flow cytometric analysis of the cultures treated with different agents. **A:** Untreated controls. **B:** UVC (50 Jm^{-2}). **C:** CHX (10 µg/ml). **D:** ActD (1 µM). **E:** Cisplatin (5 µg/ml). The different subpopulations of cells were R_1, viable cells; R_2, apoptotic cells; R_3, necrotic cells.

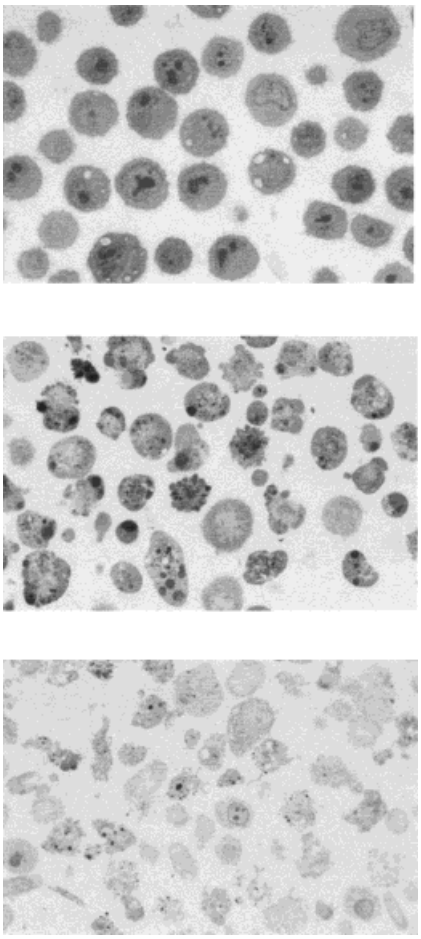

Fig. 2. Micrograph of HeLa cell subpopulations collected after sorting from cultures exposed to 50 Jm^{-2} UVC. The cells were collected from the three regions highlighted above and fixed, sectioned, stained, and photographed under light microscopy using oil immersion as described under Materials and Methods. **A:** Viable cells obtained from region R_1. **B:** Apoptotic cells obtained from region R_2. **C:** Necrotic cells obtained from region R_3.

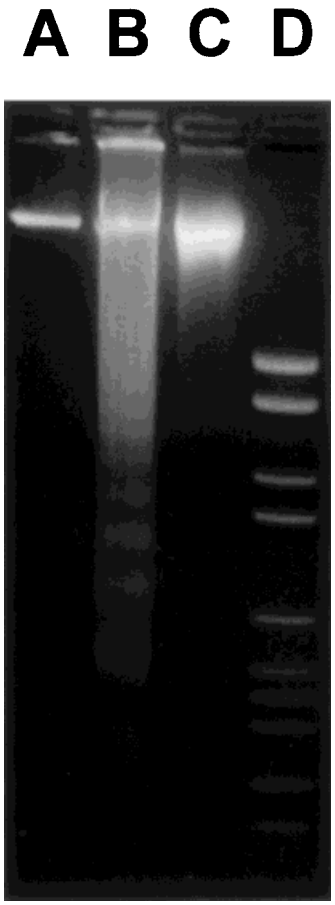

Fig. 3. Electrophoresis of the DNA isolated from HeLa cell subpopulations collected after sorting. The DNA was extracted from the sorted cells, run on a 1.5% agarose gel, and photographed as described under Materials and Methods. **A:** Viable cells obtained from region R_1. **B:** Apoptotic cells obtained from region R_2. **C:** Necrotic cells obtained from region R_3. **D:** DNA standard markers.

The effects of dose variation of different perturbants on the proportion of the three subpopulations in HeLa cultures were examined in an effort to find the concentration of agent necessary to permit optimal collection of viable, apoptotic and necrotic cells for subsequent assays. The dose effect of the different agents on the composition of the HeLa cell populations, 20 h post-treatment, can be seen in Figure 4. The dose of UVC required to produce enough cells of an apoptotic population for assay was 50 Jm^{-2}. The concentrations of the other agents that produced adequate numbers of the three cell subpopulations were as follows: CHX 10 μg/ml, Cisplatin 5 μg/ml and ActD 1 μM.

The amount of protein in the three cell subpopulations was found to differ depending on the agents used, as shown in Table I. The levels of protein found in the apoptotic cell populations were less than those of the co-treated viable cell fraction (significantly at $P < 0.05$ or $P < 0.0125$, except for ActD mainly because the ActD viable cell protein content decreased to 74% of the control cell level) while that of the necrotic cells was very much less ($P < 0.005$) in all groups. The levels of protein in viable cells from cells exposed to CHX and ActD were significantly less than those of the untreated controls ($P < 0.0125$, as determined by Bonferroni's adjustment for multiple comparisons using Student's t-test). However, the most remarkable feature of the protein changes was the constancy of the proportions of cell protein of the apoptotic (80.8 ± 1.2%) and necrotic (37.6 ±

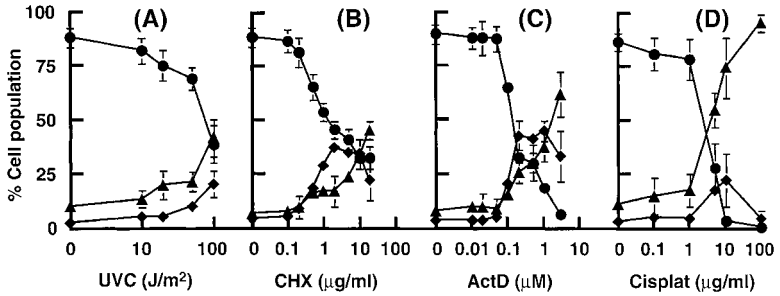

Fig. 4. Dose response of different agents on the composition of HeLa cell subpopulations as measured by flow cytometry. HeLa cells were exposed to the various agents for 20 h before analysis by flow cytometry. Before analysis, the cells were stained with H33342 and PI, as described in Fig. 1. The percentages of the subpopulations were calculated where the total population represented 100%,, and are composed of viable (●), apoptotic (♦), and necrotic (▲) cells. The cells were treated with the following agents: (**A**) ultraviolet C (UVC), (**B**) cycloheximide (CHX), (**C**) actinomycin D (ActD), and (**D**) Cisplatin. Results expressed are the mean value of 5–15 samples, while the error bars represent 95% confidence intervals.

TABLE I. Effect of Different Agents on the Cellular Protein Content of Sorted Populations of HeLa Cell Monolayer Cultures*

Agent (no. of cultures)	Cell protein (mg/10^7 cells) of subpopulations				
	Viable (V)	Apoptotic (A)	Ratio A/V	Necrotic (N)	Ratio N/V
Control (42)	6.02 ± 0.16	4.78 ± 0.14[d,e]	0.79	2.22 ± 0.10[d]	0.37
UVC (50 Jm^{-2}) (36)	5.62 ± 0.14	4.20 ± 0.18[d]	0.75	2.14 ± 0.12[d]	0.38
CHX (10 μg/ml) (26)	5.12 ± 0.36[a]	3.98 ± 0.46[b]	0.78	1.94 ± 0.12[d]	0.38
ActD (1 μM) (12)	4.46 ± 0.40[a]	4.08 ± 0.34	0.91	1.66 ± 0.12[a,d]	0.37
Cisplatin (5 μg/ml) (9)	5.80 ± 0.30	4.68 ± 0.34[c]	0.81	2.20 ± 0.28[d]	0.37

*Monolayer cultures of HeLa cells were exposed to the following agents for 20 h before being stained with H33342 and PI and sorted by flow cytometry. The statistical significance of the difference between the level of protein in the control population and the treated groups (between groups) are represented as [a]$P < 0.0125$ (calculated using Bonferroni's adjustment for multiple comparisons with Student's *t*-test), while the differences between the viable versus the apoptotic or necrotic populations within treatment groups are represented as [b]$P < 0.05$, [c]$P < 0.0125$, and [d]$P < 0.005$.
[e]n = 10 samples.

0.1%) cell subpopulations relative to their viable cohort (Table I).

Ecto-Aminopeptidase Activity

In order to evaluate the possible effects of interaction of the various ectopeptidases (Table II) of intact cells on the products of the action of each other from the common starting substrate, P9, the peptidase assays were performed both in the presence and in the absence of the aminopeptidase inhibitor bestatin [Suda et al., 1976]. This was because aminopeptidase (1) is the most active peptidase found on the intact cell surface (Table III and Fig. 5, No Bestatin), and (2) can cleave P1 from the peptide products of other ecto-peptidases, as can be seen from the large increases in P2 (YV) and P3 (YVA) production after the addition of bestatin (Table III and Fig. 5). The effect of the different perturbant agents on the activity of the cell surface aminopeptidase (eAP) in the cell subpopulations can be summarized:

TABLE II. Cleavage Products of P9 as a Result of Peptidase Activity Occurring on the Surface of Intact HeLa Cells[a]

P9-derived peptide fragment	Peptide sequence	Candidate peptidase responsible for the cleavage product
P1	Y	Aminopeptidase
P2	YV	Dipeptidase
P3	YVA	Tripeptidase
P5	YVAAA	TGFαase
P9	YVAAAVVSH	

[a]Data obtained from Brown et al. [1992].

1. Compared with those of untreated viable cells, the rates of P1 formation in viable cells obtained from cultures treated with UVC and CHX were significantly higher (in-

TABLE III. Effect of Apoptotic Agents on Cell Surface Peptidase Activities in Different Cell Subpopulations*

Apoptosis-inducing agent	FACS-sorted subpopulation	P9-derived peptide fragments (pmol/mg cell protein/15 min)							
		No Bestatin				10 μM Bestatin			
		P1	P2	P3	P5	P1	P2	P3	P5
Control (4–20)	Viable (20)	127.0 ± 5.9	14.2 ± 1.6	3.6 ± 0.4	4.3 ± 0.6	19.7 ± 1.7	37.9 ± 3.5	42.7 ± 3.1	6.6 ± 0.7
	Apoptotic (4)	252.5 ± 26.8^{d}	15.9 ± 1.0	3.7 ± 1.5	5.9 ± 0.6	26.3 ± 3.7	57.0 ± 4.0^{d}	97.8 ± 7.2^{d}	12.7 ± 2.0^{c}
	Necrotic (14)	116.7 ± 6.4	9.6 ± 0.9^{c}	9.3 ± 0.5^{d}	7.6 ± 0.8^{d}	21.4 ± 0.7	12.7 ± 1.7^{d}	30.8 ± 1.9^{d}	11.9 ± 0.4^{d}
UVC (12) ($50\ Jm^{-2}$)	Viable	148.4 ± 6.6^{f}	12.6 ± 1.3	3.9 ± 0.4	8.9 ± 1.2^{h}	10.1 ± 1.0^{h}	19.4 ± 0.9^{h}	31.8 ± 2.3^{g}	13.2 ± 0.7^{h}
	Apoptotic	231.3 ± 12.9^{d}	12.2 ± 0.9^{g}	7.3 ± 1.0^{d,e}	12.0 ± 0.9^{a,h}	17.8 ± 1.3^{d,g}	38.2 ± 2.8^{d,e}	68.7 ± 5.6^{d,e}	17.4 ± 0.7^{d,g}
	Necrotic	17.3 ± 1.8^{d,h}	6.1 ± 0.5^{d,h}	10.1 ± 0.8^{d}	11.9 ± 0.5^{b,h}	14.2 ± 1.0^{d,h}	8.1 ± 0.8^{d,f}	22.6 ± 1.7^{d,h}	12.9 ± 0.4^{e}
CHX (10) (10 μg/ml)	Viable	156.0 ± 9.1^{h}	12.2 ± 1.4	6.0 ± 0.9^{h}	7.0 ± 0.6^{h}	14.0 ± 1.9^{f}	16.4 ± 2.9^{h}	42.3 ± 4.1	7.3 ± 1.2
	Apoptotic	293.2 ± 15.8^{d}	13.5 ± 1.5	6.1 ± 0.7	10.8 ± 1.2^{c,f}	21.1 ± 2.5^{b}	62.6 ± 7.1^{d}	98.8 ± 8.0^{d}	13.1 ± 0.9^{d}
	Necrotic	8.1 ± 1.7^{d,h}	4.9 ± 0.7^{d,h}	5.6 ± 0.8^{h}	7.0 ± 1.0	6.4 ± 0.9^{d,h}	5.0 ± 0.7^{d,h}	7.6 ± 0.7^{d,h}	6.9 ± 0.8^{h}
ActD (8) (1 μM)	Viable	140.4 ± 5.0	13.4 ± 1.1	9.5 ± 1.6^{h}	10.2 ± 1.0^{h}	18.2 ± 2.7	16.5 ± 0.7^{h}	37.0 ± 3.9	10.4 ± 1.8^{f}
	Apoptotic	211.2 ± 15.3^{d}	14.3 ± 0.9	6.2 ± 0.7^{a}	11.2 ± 1.0^{h}	21.4 ± 2.6	70.1 ± 14.4^{d}	61.1 ± 4.5^{d,h}	13.8 ± 1.0
	Necrotic	9.3 ± 1.0^{d,h}	4.6 ± 1.2^{d,h}	3.8 ± 0.7^{d,h}	8.8 ± 1.1	7.5 ± 1.3^{d,h}	2.6 ± 0.1^{d,h}	9.2 ± 1.2^{d,h}	6.1 ± 1.1^{a,h}
Cisplatin (5) (5 μg/ml)	Viable	121.0 ± 10.3	6.4 ± 0.9^{g}	2.4 ± 0.2	6.6 ± 1.0^{e}	8.1 ± 1.4^{h}	12.9 ± 2.5^{h}	28.5 ± 3.2^{f}	7.2 ± 1.0
	Apoptotic	128.5 ± 12.1^{h}	13.5 ± 0.7^{d,g}	4.2 ± 1.0	13.2 ± 1.0^{d,h}	27.1 ± 2.4^{d}	57.0 ± 5.6^{d}	67.3 ± 5.0^{d,h}	9.6 ± 1.7
	Necrotic	19.5 ± 2.8^{d,h}	2.5 ± 0.3^{d,h}	8.3 ± 0.1^{d}	13.1 ± 0.9^{d,h}	4.7 ± 0.3^{h}	3.7 ± 0.7^{c,h}	21.8 ± 3.4^{f}	17.5 ± 2.1^{d,h}

*HeLa cells grown in monolayer culture were exposed to the following agents for 20 h before being stained with H33342 and PI and sorted by FACS. Cells were washed and then assayed (50,000 per well) with 10 pmoles ^{125}I-P9 for 15 min. After 15 min, the medium was removed and an aliquot spotted on a TLC plate for separation of the product peptides. Results represent the means ± SEM of 4–20 separate experiments, as shown in parentheses. The probability that such a difference between the compared values could occur by chance in viable versus apoptotic or necrotic populations within a treatment group is represented as $^{a}P < 0.05$, $^{b}P < 0.025$, $^{c}P < 0.01$, and $^{d}P < 0.005$, while the significance of differences between the same subpopulations of control and treated groups are represented as $^{e}P < 0.05$, $^{f}P < 0.025$, $^{g}P < 0.01$, and $^{h}P < 0.005$.

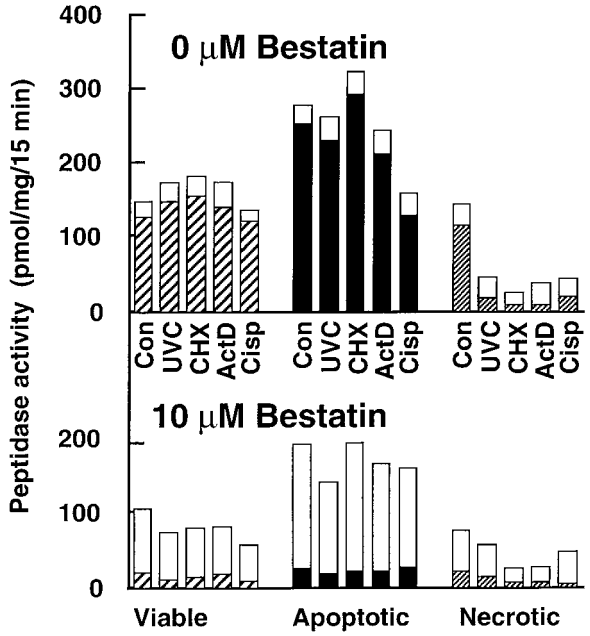

Fig. 5. Comparison of the rate of total ecto-peptidase and ecto-aminopeptidase activity in subpopulations of HeLa cells treated with different agents. The level of ecto-aminopeptidase activity on cells treated with different agents observed in the viable (wide cross-hatched bar), apoptotic (solid bar), and necrotic (cross-hatched bar) subpopulations. Total ecto-peptidase activity observed in the different treated cell subpopulations is represented as (white area in each bar). Con, control; Cisp, Cisplatin; UVC, ultraviolet C; CHX, cycloheximide; ActD, actinomycin D.

creased to 117% [$p<0.01$] and 123% [$p<0.005$], respectively) although not significantly with ActD (increased to 111%) (Table III). The increases in P1 formation were accompanied by overall increases in total cell surface peptidase activity of UVC, CHX and ActD treated cells (Fig. 5: 149 vs 174, 181, and 174 pmol/mg cell protein/15 min, respectively), with the increase in eAP activity, as a fraction of the total cell surface peptidase activity, remaining much the same as that seen in the viable population of untreated cells (85% vs 85%, 86%, and 80%, respectively).

2. It can be seen in Table III that there was no significant difference in the level of eAP activity (P1) between viable and necrotic cells of the untreated control population, whereas with cells exposed to the perturbant agents, the amount of P1 formed from P9 by the necrotic cells was much less ($P <$ 0.005 in all cases), being reduced to 5–16% of that formed by the viable cells. The percentage of total cell surface hydrolysis of P9 attributable to eAP activity fell from 80–90% in viable cells to 28–45% in necrotic cells from treated cultures (Fig. 5).
3. eAP activity was elevated in apoptotic cells compared with viable cells in the untreated controls (increased to 199% of viable cells, $P < 0.005$), as it was in cultures exposed to UVC, CHX, and ActD (increased to 156%, 188%, and 150% of treated viable cells, respectively with $P < 0.005$ in all cases, Table III).
4. However, in Cisplatin-treated cultures, eAP activity was unaltered in apoptotic or viable subpopulations, as compared with control viable cell levels, and their apoptotic cells showed no increase compared with their cohort of viable cells (Table III).

Thus, eAP activity increased in the viable cells of treated cultures (other than with Cisplatin). Necrotic cells lost most of their eAP activity in all the treated cultures, but this did not occur in control cultures. Apoptotic cells had very significant increases of eAP activity compared with viable cells in all cultures except those treated with Cisplatin.

Ecto-Di- and Tri-peptidase Activity

1. The addition of bestatin to the assays led to a reduction in the total ectopeptidase activity (ΣP1,2,3,5) of the viable and apoptotic subpopulations of all groups, with the exception of the apoptotic cells obtained from Cisplatin-treated cultures, while the total activity of necrotic cells of all the treated (but not the control) cultures did not change significantly (Fig. 5). Most of this bestatin-related loss of activity was due to the inhibition of aminopeptidase activity, since the activity of the other ecto-peptidases increased markedly (Table III), especially those responsible for the formation of P2 by ecto-dipeptidase (eDP) and P3 by ecto-tripeptidase (eTP) activity, in viable and apoptotic cells of all groups (control and treated; 1.3- to 5-fold and 4- to 26-fold, respectively).
2. In the presence of bestatin, the eDP and eTP activities more than doubled (1.97–4.42 and 1.65–2.36, respectively) with all treatments including Cisplatin, in the apoptotic cells, as compared with the viable cells (A/V) from these cultures (Table III). However, the absolute activity of the eDP of viable cells in all the treated groups fell to 34–51% of control viable cells, while that of eTP activity did not change, except for UVC and Cisplatin-

treated cells, which were reduced to 75% and 67% of control values, respectively.

Thus, compared with viable cells within a group, all treatments resulted in more than doubling of the eDP and eTP activities of the apoptotic subpopulation, but caused a small decrease in the absolute values of eTP and a larger fall in eDP of the treated viable cells, as compared with control viable cells.

Cell Surface TGFαase Activity

1. While the "TGFαase" activities (P5 production [YVAAA]) of apoptotic cells in the UVC, CHX, and ActD sets were higher than their viable cell cohorts by at least 1.3-fold (Fig. 5), only the group exposed to UVC had a significantly higher absolute TGFαase activity than the control apoptotic cells (Table III) (1.37-fold; $P < 0.01$), some 2.63-fold ($P < 0.005$) greater than that of control viable cells. The increase in TGFαase activity in the apoptotic fraction of the untreated control population compared with its viable fraction was almost 2-fold (Table III). Curiously, the necrotic cells from Cisplatin-treated cultures had as high an increase in the absolute rate of P5 production as did the UVC apoptotic population; both increased nearly 3-fold (2.65- and 2.64-fold, respectively, Table III, 10 μM Bestatin) over the value of the untreated, viable cells.
2. The bestatin enhancement of activity was smaller for "TGFαase" activity (Table III) in control and UVC groups, and was absent from the other perturbed cultures (CHX, ActD, and Cisplatin). This may reflect important differences regarding peptide substrate specificity between the P2/P3 and the P5 peptides for the eAP.

Thus, TGFαase activity was increased in apoptotic cells in all groups relative to their viable cells, with the UVC-treated apoptotic cells highest. TGFαase values of necrotic cells were higher than controls in the UVC- and Cisplatin-treated groups, but the same with CHX and ActD treatment. Bestatin increased TGFαase values only in control and UVC-treated cultures.

Ectopeptidase Inhibition Study

Activation of proteolytic activity is an integral part of the pathways effecting apoptosis. It was therefore important to establish that the changes observed in ectopeptidase activity were not attributable to the caspase-1 (ICE) activity. ICE is responsible for pro-IL-1β cleavage/activation, but it is the only cell surface caspase so far recognized and, despite the bulk of the proenzyme having a cytoplasmic location, appears only remotely involved in initiating apoptosis (e.g., [Cohen, 1997; Cryns and Yuan, 1998]). Table IV shows the data from studies using caspase-1 and metalloprotease inhibitors on cell surface peptidase activity in apoptotic and viable cell populations from CHX- and UVC-treated cell cultures. None of the cell surface ectopeptidases in the apoptotic population of CHX-treated cultures was inhibited by the caspase-1 inhibitor Ac-YVAD-CHO, which inhibited ICE activity 60.0 ± 2.7% (SEM; n = 9) in separate experiments. The inhibition results were similar with control and ActD subpopulations (data not shown) and suggest that activation of the cell surface ectopeptidases are not due to increased caspase-1 activity.

The eAP, eDP, and TGFαase found on the surface of apoptotic cells in CHX-treated cultures were significantly inhibited by O-phenanthroline in the presence or absence of bestatin (Table IV). The anomalous stimulation of eTP in the presence of O-phenanthroline, but in the absence of bestatin, is probably due to the much greater inhibition of eAP than eTP by the Zn^{2+} chelator. This would allow greater accumulation of P3, which seems to be more susceptible to eAP cleavage than the other peptides, as is apparent from the bestatin studies listed in Table IV, demonstrating very large increases in P3 production relative to other peptides products. This view is supported by the inhibition of the eTP stimulation by O-phenanthroline in the presence of bestatin. The remarkable similarity in the degree of inhibition of the peptide cleavage with either inhibitor in both viable and apoptotic cells suggests that the different levels of activity in both subpopulations resulting from CHX (and other) treatments are due to changes in the same ectopeptidases. Clearly, the ectopeptidase activities identified by this peptidase assay belong to the metalloprotease family and are all increased in apoptotic cells whether resulting from "natural" causes (i.e., in control cultures) or from "genetic stress" induced by a variety of agents. They are not, however, increased in viable or necrotic cells in these cultures.

TABLE IV. Effect of Protease Inhibitors on Cell Surface Peptidase Activity of Viable and Apoptotic Cells from CHX or UVC-Treated HeLa Cultures*

Cell subpopulation	Inhibitor	P9-derived peptide fragments (pmol/mg cell protein/15 min)							
		No Bestatin				10 μM Bestatin			
		P1	P2	P3	P5	"P1"	P2	P3	P5
CHX-treated cultures									
Viable	Control	145.3 ± 5.1	11.2 ± 2.3	4.0 ± 1.1	7.3 ± 0.7	12.1 ± 1.9	15.3 ± 1.6	45.4 ± 4.9	6.9 ± 1.0
	O-Phenanthroline	6.0 ± 2.4[c]	1.7 ± 0.7[c]	6.3 ± 2.4	0.8 ± 0.3[c]	3.2 ± 1.2[c]	4.4 ± 2.7[b]	23.0 ± 4.9[b]	1.4 ± 0.3[c]
	Ac-YVAD-CHO	139.6 ± 7.3	9.4 ± 1.1	5.8 ± 0.6	7.8 ± 2.8	10.2 ± 2.2	13.4 ± 0.8	43.9 ± 5.4	7.0 ± 0.7
Apoptotic	Control	305.8 ± 12.1	14.1 ± 3.7	5.5 ± 1.3	10.0 ± 1.0	17.3 ± 1.1	69.1 ± 6.5	96.7 ± 7.2	14.0 ± 4.1
	O-Phenanthroline	15.6 ± 4.4[c]	2.6 ± 0.7[c]	11.6 ± 2.7[a]	1.7 ± 0.4[c]	3.5 ± 0.6[c]	2.6 ± 0.9[c]	46.9 ± 8.9[c]	1.9 ± 0.5[c]
	Ac-YVAD-CHO	288.8 ± 19.7	12.8 ± 4.5	5.6 ± 1.4	9.6 ± 0.8	19.1 ± 2.0	66.0 ± 5.6	102.5 ± 12.2	12.5 ± 4.6
UVC-treated cultures									
Viable	Control	152.4 ± 16.3	14.2 ± 3.5	2.9 ± 0.5	7.2 ± 0.8	22.6 ± 2.6	43.6 ± 2.8	60.8 ± 7.7	14.6 ± 1.5
	O-phenanthroline	26.3 ± 6.6[c]	2.2 ± 0.5[c]	7.5 ± 2.0[a]	1.8 ± 0.6[c]	6.5 ± 1.4[c]	2.8 ± 0.7[c]	6.5 ± 1.4[c]	2.2 ± 0.6[c]
	Ac-YVAD-CHO	154.2 ± 17.2	10.0 ± 3.0	2.8 ± 0.9	6.4 ± 0.6	20.7 ± 4.8	37.9 ± 3.9	59.8 ± 6.3	14.1 ± 1.2
Apoptotic	Control	228.1 ± 21.1	12.7 ± 1.2	7.1 ± 2.6	11.6 ± 1.3	20.7 ± 2.2	40.8 ± 2.6	70.8 ± 9.2	15.8 ± 1.0
	O-phenanthroline	23.1 ± 7.4[c]	2.7 ± 0.8[c]	17.7 ± 8.7	2.3 ± 0.6[c]	6.3 ± 2.7[c]	3.6 ± 1.3[c]	27.1 ± 5.2[c]	2.5 ± 0.2[c]
	Ac-YVAD-CHO	238.8 ± 18.1	11.8 ± 1.4	7.4 ± 1.8	11.5 ± 0.5	20.3 ± 3.6	40.4 ± 7.9	67.2 ± 15.7	14.6 ± 1.1

*HeLa cells grown in monolayer culture were exposed to CHX (10 μg/ml) or UVC (50 Jm^{-2}) for 20 h prior to being stained with H33342 and PI and sorted by FACS. Cells were washed and resuspended in HBSS and placed in a 37°C incubator for 15 min before being exposed to O-phenanthroline (0.25 mM) or Ac-YVAD-CHO (1 μM). At the end of this period, 10 pmoles ^{125}I-P9 was added to the cells for 15 min. After this time, the medium was removed and an aliquot spotted on a TLC plate. Results represent the means ± SEM of four separate experiments. The statistical significance of the difference between the inhibitors and control values is represented as [a]$P < 0.05$, [b]$P < 0.01$ and [c]$P < 0.005$.

DISCUSSION

Stimulated by our previous observations [Ellem et al., 1988] that TGFα was released as a result of proteolytic cleavage [Lee et al., 1995] from its surface membrane-bound precursor form, after UVC exposure of several cell strains/lines derived from epithelial sources, we sought to identify the enzyme responsible and for evidence that regulation of its activity was causal in TGFα release. Using the peptidase assay underpinning the present study, we showed that several agents capable of inducing "genetic stress" [Herrlich et al., 1986] caused an increase in an ectopeptidase activity that was a candidate "TGFαase" in HeLa cultures [Brown et al., 1993]. In addition, genetic stress was shown to be accompanied by activation of at least three other ectopeptidases (eAP, eDP, and eTP) in those experiments. The activity of these cell surface ectopeptidases was shown to be increased by inhibitors of RNA and protein synthesis [Chenevix-Trench et al., 1992], as in the present study. It was subsequently found that the degree of increase in the ectopeptidase activity of these cultures was correlated with the level of occurrence of apoptosis therein [Brown et al., 1994].

The present study showed that the total and individual ectopeptidase activities of the apoptotic subpopulation of cultured cells were nearly 2-fold greater than those of viable cells. The major changes in activity amongst the ectopeptidases of the apoptotic cells varied with the treating agent. Maximum increases were seen in the eDP (~4-fold) of CHX-, ActD-, and Cisplatin-treated cultures and eTP (>2-fold) in control, UVC-, CHX-, and Cisplatin-treated cultures. The range of increases in TGFαase activity of apoptotic cells was 1.3–1.9-fold greater than that of the viable cells from the same culture. There does not appear to be a global cellular response, nor is there an "across-the-board" increase of similar magnitude for each proteolytic activity.

These data are directly relevant to the issues arising from the use of cell protein as the normalizing variable rather than cell number. Table I shows that 20 h of exposure to either of the macromolecular synthesis inhibitors CHX or ActD caused the cellular protein content of the viable cell subpopulation to fall significantly, as compared with control cultures, whereas UVC and Cisplatin treatment did not. Of much greater magnitude were the decreases observed when the protein contents of apoptotic (20%) or necrotic cells (62%) were compared with those of the viable cell subpopulations. Darzynkiewicz et al. [1992] also observed a marked diminution of protein content in flow cytometry analyses of apoptotic and necrotic cells. Simultaneously, equivalent variation in all the activities would favor a global "artifact" (e.g., due to generic surface area changes in relation to cell volume, or to the flip-flop of phospholipid orientation in the cell membrane), whereas non-equivalent or differential changes would suggest that these modulations resulted from individual, functional, peptidase variation, independent of cell surface area alterations.

The increase in ectopeptidase activity in apoptosis cannot be explained by protein loss, per se, as this would cause an increase of only 1.25-fold; thus, it must represent some form of activation of their function, which, if generic, probably exerts differential effects through individual ectoenzyme characteristics. The decrease in necrotic cell ectopeptidase activity (Table III) of the treated cultures is undoubtedly due to (1) the loss of cell membrane components (Fig. 2), and (2) leakage of the intracellular, soluble contents with rapid decline in the amount of protein associated with the cell carcass. Brown et al. [1994] showed that eAP is the main ectopeptidase shed from necrotic (Trypan blue-stained) HeLa cells that had lost cell adherence, but not from viable adherent cells, in cultures treated with a wide range of agents, including UVC, CHX, and ActD. eAP does not appear to be shed from control necrotic cell populations, so the shedding process may only occur when cells are exposed to lethal levels of genotoxic agents. Second, if the cells had not lost intracellular proteins, one would expect that as the plasma membrane becomes permeable during necrosis, P9 could be processed by intracellular ectopeptidases. This does not seem to be the case, as the intracellular ectopeptidases constitute 99% of the total cellular ectopeptidase activity [Brown et al., 1992] and, if some were present, the amount of P9 cleaved by the necrotic cells would be considerably higher than that observed (Table III). While the fall in necrotic cell protein could increase enzyme activities associated with this residue, all the activities fell when normalized on a protein basis, with the exception of TGFαase. In this regard, it is of interest that necrotic cell

TGFαase was diminished least, or not at all, of the ectopeptidases of all cultures and was found to be the most tightly bound ectopeptidase during cell membrane purification in our previous study [Piva et al., 1997]. Thus, differential retention of enzymatic activity during cellular disintegration may well be an important means whereby the enzymatic activity can be increased at the site of cellular debris without evoking specific enzyme activation, and may contribute to the cleanup operation needed to remove the detritus and restore the normal tissue milieu.

The twofold increase in eAP activity (P1 production in the absence of bestatin) of apoptotic cells compared with that of viable cells in control cultures in Table III appears to be unique, as products P2, P3, P5 were not significantly different from the control values. However, examination of Table III shows that if the activities of eDP and eTP are used when assayed in the presence of bestatin so that their values will not be corrupted by the activities of eAP, then, for control cultures, the ratios of the apoptotic to viable cells (A/V) show a factorial increase of approximately twofold in all of the ectopeptidases, including eAP (assayed without bestatin) (data calculated from Tables I and II). This would seem to favor a generic "artifact," although the ratios of the ectopeptidase activities of the necrotic to viable (N/V) cells vary from 0.34 to 1.8 (data calculated from Tables I and II). Also, the values for the A/V ratios of the treated cultures ranged within 1.32–2.16, 1.79–3.82, 1.33–4.25, and 1.33–4.42, for UVC, CHX, ActD, and Cisplatin, respectively, indicating the likelihood that individual ectopeptidase change resulted from unique rather than global ectopeptidase responses. In a manner consistent with this latter interpretation, the individual N/V values also show considerable variation (0.34–1.80, 0.12–0.98, 0.05–0.95, 0.07–0.59, and 0.16–2.43 for control cultures and those treated with the same series of perturbants, respectively).

Since apoptosis is accompanied by a series of proteolytic events concerned with disabling the cell functions normally involved in homeostasis and repair, the question arose as to whether the increased ectoprotease activity was related to these intracellular events. We have shown that the ectopeptidases are metalloenzymes while the proteases involved with the induction of apoptosis are caspases [Cohen, 1997; Nicholson and Thornberry, 1997; Cryns and Yuan, 1998; Porter and Jänicke, 1999] that cleave many intracellular proteins whose partial hydrolysis cements cell death. Caspase-1 inhibitors failed to inhibit the activity of ecto-metallopeptidases in this study (Table IV). At present there are no data to establish a mechanistic connection between these two protease activation events or between the processes stimulating the calpains [Squier and Cohen, 1996] and serine proteases [Sukharev et al., 1997] that are also associated with the generation of apoptosis.

Ecto-metalloprotease activity was responsible for the shedding of CD16 from neutrophils [Middelhoven et al., 1997] and CD13 from THP-1 monocytes undergoing apoptosis [Brown et al., 1996]. It is unknown whether the activity of other metalloproteases is affected during apoptosis. However, shedding of membrane-anchored growth factors and receptors, other ectoenzymes, cell adhesion molecules, and a variety of proteins of unknown significance has been reviewed previously [Ehlers and Riordan, 1991; Arribas et al., 1996; Hooper et al., 1997] and autocrine, paracrine, and even endocrine roles for the shed bioactive molecules have been noted. The roles played by these membrane proteases in the triggering or modulation of the mechanisms of local inflammatory and reparative activities in stressed tissues remains to be investigated.

Among the ecto-metalloproteases, eAP was found to be the most active on the P9 substrate, accounting for up to 90% of total peptidase activity in some cell subpopulations. It has been suggested that eAP may be responsible for the cleavage of amino acids from peptides in the extracellular fluids, which would permit the cells to recover essential amino acids via a salvage type mechanism [Plakidou-Dymock et al., 1993; Piva et al., 1998]. There is a possibility that the loss of eAP activity observed in necrotic cells (Table III and Fig. 5) is due to shedding from the cell membrane. Brown et al. [1996] found that. in THP-1 cells. aminopeptidase N was shed from the cell surface as the cell underwent apoptosis. While necrotic cells from control cultures had no loss of activity (Table III) the treated groups had a decline in eAP activity to 5–16% of that of their cohort's eAP, a difference for which we have no obvious explanation. We presume that different modes of necrotic exitus may involve different degrees of membrane dissolution and thus loss of ectoproteases.

A possible role for eAP as a surface molecule or as a shed, matrix-bound molecule is to contribute to the dissolution of matrix components to enhance local cell mobility and thus access to the "apoptotic shrapnel" for phagocytic removal [Menrad et al., 1993; Saiki et al., 1993; Brown et al., 1996]. Matsunaga et al. [1996] found that rotenone treatment of HL-60 cultures, which induced some 40% apoptosis over several days' exposure, resulted in a significant increase in CD13 and CD38 expression, which was apparently global, in all the cells.

It has been suggested by various investigators that there may be more than one form of non-necrotic cell death [Schwartz, 1995; Zakeri et al., 1995]. Without entering this debate, we wish to emphasize that, on the basis of our studies, there may well be modifications of the cellular phenotype of the same cell type dying a non-necrotic death by a process that follows a typical apoptotic morphological pattern, when induced by different perturbants, and that such modifications may have subtle consequences for the in vivo reactions thus provoked.

While the principal finding of this study is that the activation of the metallopeptidase set of ectopeptidases during apoptosis could be one of the committal steps locking a damaged cell into programmed death, rather than recovery, details in the differences observed remain unexplained but may reflect the characteristic fingerprints of the process of cell death.

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