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Low Fluences of Ultraviolet Irradiation Stimulate HeLa Cell Surface Aminopeptidase and Candidate "TGFαase" Activity

S.B. Brown, D. Krause, and K.A.O. Ellem

Queensland Cancer Fund Research Unit, Joint Experimental Oncology Programme, Queensland Institute of Medical Research, The Bancroft Centre, Brisbane, Queensland, 4029 Australia

Abstract Several forms of perturbation result in the release of bioactive molecules into the microenvironment of injured cells to mediate the inflammatory or reparative reactions which restore normal tissue structure and function. Amongst other products, ultraviolet irradiation (UV) causes the release of the growth factor TGFα from a variety of epithelial cell sources, apparently by a post-translational mechanism. Here we have explored the hypothesis that UV results in the activation of cell surface proteases which may then be capable of excising mature TGFα from its plasma membrane-bound precursor. Using a recently described, sensitive assay of peptidase activity tailored to the substrate requirements for cleavage of the scissile bonds in proTGFα, we have found that nonlethal fluences of UV ($< 12\ Jm^{-2}$) to HeLa cell cultures are followed by large increases in cell surface proteolytic activities. Amongst these, endopeptidase activity produces a similar product profile from the nonapeptide substrate to that of human leukocyte elastase, an enzyme previously shown to be capable of releasing a bioactive, mature form of TGFα from its cell-bound precursor. However, in addition to this candidate "TGFase" activity, cell surface aminopeptidase activity was also very significantly increased. The increase in the two classes of peptidase function differed in the timing of their responses. Aminopeptidase activation occurred immediately following UV, peaking after some 15–20 h, whereas the increase in endopeptidase activity lagged 6 h behind, cresting after 20–24 h. No evidence for a role for aminopeptidase in the activation of the endopeptidase could be found. Also, there was no increase in the total proteolytic activity demonstratable in cell extracts following UV.

Attempts to interrupt the UV peptidase activation by inhibiting protein synthesis with cycloheximide were unsuccessful; rather, the inhibitor itself caused an increase in both classes of peptidase activity during the first 20 h. Unlike the UV response, both the aminopeptidase and endopeptidase ectoactivities increased simultaneously within a few hours of introducing cycloheximide into the medium of unirradiated cultures. The cycloheximide induced activity peaked after 20 h. Interestingly, cycloheximide alone has previously been shown to potentiate TGFα release from a cell line producing its precursor constitutively.

These data suggest that both UV and cycloheximide can initiate reactions in HeLa cells which result in ectopeptidase activation of a global nature. Since both agents result in rapid interruption of DNA synthesis, it is possible that this cell surface proteolytic response may be analogous to, or part of, the "mammalian genetic stress response." The mechanism of the activation of the cell surface proteases appears to be post-translational, perhaps part of a proteolytic cascade originating from perturbed macromolecular synthesis.

Key words: cognate peptide substrate, post-translational stimulation, cycloheximide activation, preproTGFα, bestatin, ectopeptidases, ultraviolet irradiation

The first decade of research on the epidermal growth factor (EGF) homologue, transforming growth factor alpha (TGFα), was underpinned by the assumption that this protein was an oncofoetal principle [e.g., Salomon et al., 1990] whose activity was that of an autocrine or paracrine mitotic stimulant [e.g., Sporn and Todaro, 1980]. Cloning of TGFα cDNA [Lee et al., 1985; Derynck et al., 1984] indicated that it was synthesized as a transmembrane, 160 amino acid precursor. Comparison of the amino acid sequence with that deduced from its cDNA indicated that the 50 a.a. mature form released by many tumour cell lines was cleaved from the precursor at A†V bonds set in hydrophobic, hep-

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Address reprint requests to K.A.O. Ellem, Queensland Institute of Medical Research, The Bancroft Centre, 300 Herston Road, Brisbane, Queensland, 4029 Australia.

tapeptide runs at both ends of the molecule ($A_{39}\dagger V_{40}$ and $A_{89}\dagger V_{90}$). It is presumed that in vivo an elastase-like enzyme is responsible for cleavage of TGFα from its precursor and that, under a variety of circumstances, this can result in both autocrine and paracrine mitogenesis in a physiological (tissue repair, embryonic development) or pathological (psoriasis, a variety of malignancies [e.g., Derynck et al., 1987; Gottlieb et al., 1988]) context. Certainly, processing of the precursor has been demonstrated in vitro with transfected cell systems suggesting that the basic outline of the mode of TGFα production is correct [e.g., Bringman et al., 1987; Teixidó and Massagué, 1988; Pandiella and Massagué, 1991].

Recent findings have added a new dimension of complexity suggesting that the cell surface residence of proTGFα may extend the range of functions performed by this molecule. Site-directed mutagenesis has been used to abrogate the scissile bonds in proTGFα [Brachmann et al., 1989; Wong et al., 1989] and thereby eliminate processing. The membrane anchored proTGFα is capable of stimulating EGF receptor (EGFR) mediated responses in adjacent cells in model, mixed cultures. This interaction has also been shown to potentiate cell-cell adhesion, followed by cell replication, of an EGFR expressing haematopoietic precursor cell line when added to a proTGFα carrying bone marrow stromal cell culture [Anklesaria et al., 1990]. While pro-$TGF\alpha_{160}$ has only 1/100th the mitogenic activity of $TGF\alpha_{50}$ in solution [Brachmann et al., 1989], it may still be an important modulator of cell-cell interactions concerned with replication and differentiation. Whether as a released, soluble, mature form or a membrane-bound precursor, the activity of TGFα is patently dependent on the proteolytic cleavage events which produce the released form at the expense of the plasma membrane precursor. Different cell types may be set to perform one or both types of TGFα related function. Clearly the expression of the appropriate protease activity at the cell surface is the regulating mechanism determining which activity will be in evidence.

Nothing is currently known about endogenous cellular enzymes involved in processing TGFα. It has been shown that both porcine pancreatic and human leukocytic elastases are capable of excising a 6 kD TGFα from its precursor [Mueller et al., 1990; Ignotz et al., 1986] but end terminal analysis has not yet been reported to verify that the scissile bonds are those expected ($A_{39}\dagger V_{40}$ and $A_{89}\dagger V_{90}$). The known elastase preference for A†X [e.g., Werb et al., 1982] would allow several options for a scissile bond. It is apparent that bioactivity of the various forms of TGFα (pro-, meso, mature, etc.) is not absolutely dependent on complete or even precise cleavage [e.g., Leutteke et al., 1988; Wong et al., 1989].

We have found that ultraviolet light, in doses which do not cause cell death (UVC 3–6 Jm^{-2}), results in TGFα release from cultures of normal melanocytes, most melanoma lines (whether or not they were detectably constitutive for TGFα release), and HeLa cells but not fibroblasts [Ellem et al., 1988]. We were unable to demonstrate any change in TGFα mRNA levels following UVC irradiation, nor did we find a good correlation between TGFα mRNA levels and constitutive levels of mature TGFα production. To test the hypothesis that the UVC effect might be a post-translational phenomenon we attempted to block TGFα induction with a series of inhibitors of macromolecular synthesis. Inhibitors of cytoplasmic mRNA production (5,6-Dichloro-1, β-Ribofuranosyl-Benzimidazole), total RNA synthesis (Actinomycin D), and protein synthesis (cycloheximide) were all without significant effect on the UVC-induced increase in TGFα. The combined weight of evidence indicated that the UVC effect is due to a post-translational mechanism [Chenevix-Trench et al., 1992]. Furthermore, the presence of the inhibitors themselves in the absence of UVC was responsible for increasing TGFα release, but to a lesser degree than with UVC irradiation. Since these inhibitors are followed by a secondary interruption of DNA synthesis, this suggests that the induced TGFα release may be part of a global response—perhaps akin to the "mammalian genetic stress response" (MGSR) codified by the Karlsruhe group [Herrlich et al., 1986] as a mammalian analogue of the bacterial SOS response [e.g., Walker, 1985].

The most likely post-translational process which would account for UVC stimulated release of TGFα from irradiated cells would be increased cleavage of proTGFα. This could be due to 1) activation of surface proteolytic activity (of the elastase type, say a TGFα-ase), 2) increased exposure of proTGFα to a cell surface TGFα-ase by rearrangements on the plasma membrane, or 3) conformational changes within

proTGFα resulting in a more favourable presentation of its scissile bonds to the appropriate enzyme. To examine the first hypothesis—that UVC causes activation of cell surface proteolytic activity of TGFα-ase type—we devised an assay with built-in specificity for the reaction of interest using a peptide cognate with the TGFα cleavage sequence as substrate. The assay proved to be quick, precise, adaptable to volumes as small as 5 μl, exquisitely sensitive, linear up to 70% hydrolysis of substrate (approximately first order kinetics), and cheap [Brown et al., 1992].

Using this assay we report here that low fluences of UVC (< 12 Jm^{-2}) were followed by increases up to fivefold in cell surface protease (CSPase) activity without change in total cell activity for the substrate. Not only was TGFαase-like activity increased, but also cell surface aminopeptidase activity was elevated commensurately. The time of initiation of both sets of activities was different as was the time of maximum increase. The results of inhibitor experiments suggest that the changes are post-translational and that cell surface proteases may vary in activity as part of a global response to perturbation of macromolecular synthesis.

We are thus presented with the fascinating phenomenon that intact cultures of HeLa cells show successive waves of increase in cell surface aminopeptidase and endopeptidase activity for the substrate P_9 in the 30 h following nonlethal UVC irradiation. Presumably the activation of the endopeptidase(s) is responsible for cleaving mature TGFα from proTGFα causing its release, since the spectrum of products from P_9 was the same as that produced by human leucocyte elastase, and this enzyme has already been shown to release bioactive TGFα from proTGFα [Mueller et al., 1990].

MATERIALS AND METHODS

Materials

Chloramine T, polyoxyethylene(20)sorbitan mono-oleate (Tween-80), and D-mannitol were purchased from BDH Chemicals Ltd (Poole, England); sodium dihydrogen orthophosphate, AJAX Chemicals Pty Ltd (Auburn, NSW, Australia); butan-1-ol and sodium chloride (NaCl), May and Baker Ltd (Dagenham, England); RPMI-1640 growth media, Gibco Laboratories (Grand Island, NY); suc(Ala)$_3$NA, Bachem Inc. (Torrance, CA); trypsin, penicillin and streptomycin, Commonwealth Serum Laboratories (Melbourne, Vic., Australia); Triton-X100, cycloheximide, and bestatin, Sigma (St. Louis, MO). All other reagents were of AR grade. All aqueous solvents were prepared with Milli-Q purified water.

Methods

Methods were previously described by Brown et al. [1992]. Briefly, an octapeptide was synthesized to be cognate with the N-terminal cleavage site for mature TGFα, and to which an N-terminal tyrosine was added to permit radioiodination. The iodinated nonapeptide substrate (H-YVAAAVVSH.NH_2, denoted P_9 herein) was separated from its products by TLC on silica gel plates and the R_f's of all iodinated peptides from Y, YV, YVA . . . , etc. (denoted P_1, P_2, P_3 . . .), as putative products, were determined so that the site of the scissile bonds could be assessed. Quantitation of the ^{125}I labelled substrate and products was performed by γ-counting of autoradiographically identified bands, by densitometry of autoradiograms, but most conveniently and accurately by phosphorimage analysis. P_9 in HBSS was incubated (30–120 min) at 37°C in the presence of intact monolayer cultures of HeLa cells or their extracts (5% n-Butanol or 0.05% Triton-X100). After the appropriate incubation time the supernatant assay solution was removed and placed on ice with an aliquot immediately spotted onto a multi-channeled TLC plate (Whatman, LK6D) and the remainder of the incubation mixture frozen. The fully loaded TLC plate was developed with butanol:acetic acid:water:: 100:10:30 (BAW) or butanol:acetic acid:water: pyridine::100:6:30:1 (BAWP), as appropriate, and subjected to autoradiography or exposed to storage phosphorscreen (Kodak) from which the image was realized by phorphorimager (Molecular Dynamics, Sunnyvale, CA) analysis and quantitated with ImageQuant software (version 3.15).

Cell culture. HeLa cell cultures grown in 24-well plates (Linbro, 2 cm^2) were used in all experiments. To inactivate endogenous peptidase activity in FBS, the serum was heated to 70°C for 1 h as previously described. Cells cultured for > 20 passages in RPMI-1640 supplemented with 10% serum which had been heat inactivated at 70°C (S70) are designated HeLaS70. Those with < 3 passages in S70 are designated as HeLaN70. Cultures were seeded with 20×10^4 cells/well in 1 mL of S70 media and allowed to incubate for 56 h before irradiating the cells in HBSS without phenol red. Cell

viability was determined by cell counts on replicate cultures using trypan blue 0.2% in PBS.

Ultraviolet irradiation. Medium was gently aspirated from monolayer cultures of HeLa cells in 24-well plates and overlaid with 0.5 mL HBSS prior to irradiation from a UV source (UVS-52 Minneralite lamp, Ultraviolet Products Inc., San Gabriel, CA) with a predominant output at 254 nm and less than 3% UVB. Irradiations were performed 8 wells at a time with a flux of 0.74 $Jm^{-2}s^{-1}$ or 4 wells at a time with 3.70 $Jm^{-2}s^{-1}$ for fixed times to give varying doses of total UVC. Cells were refed with fresh media and allowed to incubate for a further 20 h before assay. All plates in an experiment were irradiated within a 5 min period. The blank nonirradiated controls were sham irradiated and dealt with similarly.

The lamp flux rate was routinely determined using an IL1700 Research Radiometer (International Light Inc., MA) recently calibrated against standards traceable to the National Institutes of Standards and Technology. For determination of UVC a photodector SED240 with filter NS254 (maximum sensitivity 253.7 nm) and diffuser, and for UVB the same detector-diffuser with filters SCS280 (maximum sensitivity 287 nm) was used. The meter used in a previous publication [Ellem et al., 1988] underestimated fluences by a factor of 1.85×.

RESULTS

Effect of UVC on Cell Numbers: Multiplication and Viability

Refeeding 24-well cultures with fresh media with no prior exposure to UV irradiation resulted in a steady increase in cell numbers (Fig. 1) with a doubling time of 36 h at the relatively high densities employed (20×10^4 cells cm^{-2}, at $t = 0$). We have, however, observed that if control cultures were not refed with fresh media the resulting cell numbers increased more than those which were refed, indicating that cells experienced a slowdown in growth after a change of medium. This probably explains the linear rather than exponential rise in Figure 1. Cell numbers were always determined after an HBSS wash of the monolayer in which the remaining adherent cells, which were counted or assayed, had a low percentage which stained with trypan blue (e.g., Table I).

At doses of $\geq$ 18.5 Jm^{-2} the cell numbers did not increase over a 30 h period and the cells became noticeably more irregular in shape, even-

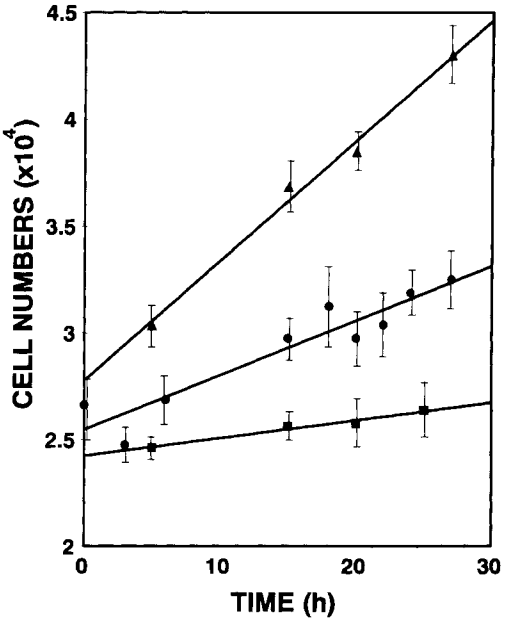

Fig. 1. Effect of UVC on cell multiplication. At zero time cells were sham irradiated (▲) or irradiated with 11.1 Jm^{-2} (●) or 18.5 Jm^{-2} (■) of UVC (0.74 $Jm^{-2}s^{-1}$) and refed with fresh media (S70) and left to incubate for varying times. Changes in cell density with time after irradiation were determined from haemocytometer counts of trypsinized well cultures (2 cm^2), each point representing the mean of 8 replicate counts. The bars represent 95% confidence intervals (C.I.).

tually detaching from the monolayer. After 45 h all cells were no longer adherent and subsequently stained with trypan blue indicating cell death. A dose of 14.8 Jm^{-2} resulted in some cells "blistering" mildly, but overall looking normal. However, we observed that after 21 h cell numbers had not significantly increased over numbers obtained at the time of irradiation (results not presented). Cell cultures irradiated with a total dose of 11.1 Jm^{-2} or less showed little morphological change under the microscope, but replicated at a slower rate relative to control growth without showing any increase in trypan blue staining (e.g., Fig. 3B). Table I confirms that the number of cells found floating increased with UV irradiation dose and that the majority of these cells stained with trypan blue. Since 10–12 Jm^{-2} of UVC was the upper limit of fluences found to stimulate fibroblast cell growth [Cohn et al., 1984] and melanocyte DNA synthesis [Ellem et al., 1988] and since it did not result in cell death of HeLa cultures, this dose of UVC was used as the standard dose in subsequent experiments.

A potentially confounding variable in studies of intact cell surface (ectopeptidase) activity is

TABLE I. Effects of UVC on Cell Growth and Viability

Dose	Total cells as % control	Nonviable cells in monolayer (%)	Cells floating (%)	Floaters not viable (%)
0	100.0	7.4	0.1	10
11.1	73.5	7.1	2.0	88
18.5	56.7	5.8	3.3	100
22.2	57.1	7.1	3.9	96
29.6	58.1	6.2	5.2	99
37	54.0	5.9	9.8	100

cell death, with loss of plasma membrane integrity and release of intracellular enzymes capable of hydrolyzing P_9—which are in much greater supply than the putative cell surface peptidases [Brown et al., 1992]. We have, therefore, been at pains to ensure that experimental conditions minimize or do not provoke cell death within the period of experimental observation.

Effect of UVC: HeLaN70 vs. HeLaS70

It was previously found that FBS had significant proteolytic activity for the substrate used in this study and that heat inactivation at 70°C for 1 h (S70) was necessary to eliminate it as a confounding factor [Brown et al., 1992]. The subculture of HeLaS56 into media containing S70 serum was at first accompanied by poor cell adhesion followed by an increased ability of the cells to attach after 3–4 passages. The growth rate of the cells remained unchanged. It was necessary, however, to show that the cellular responses of interest, viz. activation of cell surface proteases by UVC found in some preliminary experiments, was not significantly affected. We therefore determined the dose response curves for intact cell (cell surface) proteolytic activity of HeLaS70 and HeLaN70 cultures.

HeLaS70 and HeLaN70 cells seeded 56 h previously into 24-well plates were irradiated to the indicated fluence at a flux rate of 0.74 or 3.7 $Jm^{-2}s^{-1}$ and assayed 20 h later. Each point in Figure 2 represents the hydrolysis of P_9 by a single culture as determined by direct quantitation of the radioactive bands following TLC, band scraping, and γ-counting. The data in Figure 2A,B have been further corrected for variations in cell numbers using the mean cell count of 4 replicate cultures for each fluence where all points have been normalized to a cell density of 25×10^4 cells cm^{-2} (the value corresponding to 100% confluence). Results were normalized to correct for variations in cell number for cultures in which cell density varied from 12.5×10^4 cells cm^{-2} (37 Jm^{-2}) to 27×10^4 cells cm^{-2} (0 Jm^{-2}). We have previously shown that cell surface peptidase activity of unirradiated cultures was linear as a function of cell number over the range of cell densities employed [Brown et al., 1992].

As can be seen from Figure 2, HeLaN70 and HeLaS70 both responded similarly showing UVC stimulation of cell surface peptidase activity in a dose dependent manner. The greater the total dose of UVC, the greater was the increase in the level of peptide hydrolysis, although the data for fluences > 15 Jm^{-2} is distorted and underrepresented because of substrate limitation [Brown et al., 1992]. This is apparent from densitometer images of autoradiograms of the TLC plates (Fig. 2 insets) where a progressive increase in the percent hydrolysis of P_9 can be seen until almost complete, despite an accompanying decrease in cell numbers (e.g., Fig. 1). It is also clear from the insets of Figure 2 that the main product of P_9 hydrolysis is P_1 so that the major peptidase activity being measured is that of cell surface aminopeptidase. The flux rate appeared to have a small effect, since for fluences < 15 Jm^{-2} the lower rate caused a 25–50% higher peptidase activity, suggesting that over a limited range, there may be an inhibitory effect of the higher flux. Overall, and correcting for a limiting substrate assay, a greater than tenfold increase in cell surface activity towards P_9 was observed with doses up to 37 Jm^{-2}. The response of both cell populations is clearly similar and further studies were, therefore, conducted only with the better adapted HeLaS70 subline.

The cell viability, as determined by trypan blue staining, was not significantly decreased over the range of UVC fluences studied within the 20 h of the observations. This can be seen from the data in Table II in which the relevant variable for the assay—the average number of dead cells present in replicates of the cultures used for assay and treated similarly—is reported for the experimental groups generating Figure 2A,B. There is neither an increase in dead cells/assay, nor a significant increase in percentage of dead cells in the cultures associated with increased fluence.

Some interesting features of the UVC dose response curves for HeLaS70 when refined with more sampling points and less substrate hydrolysis are presented in Figure 3. We have previ-

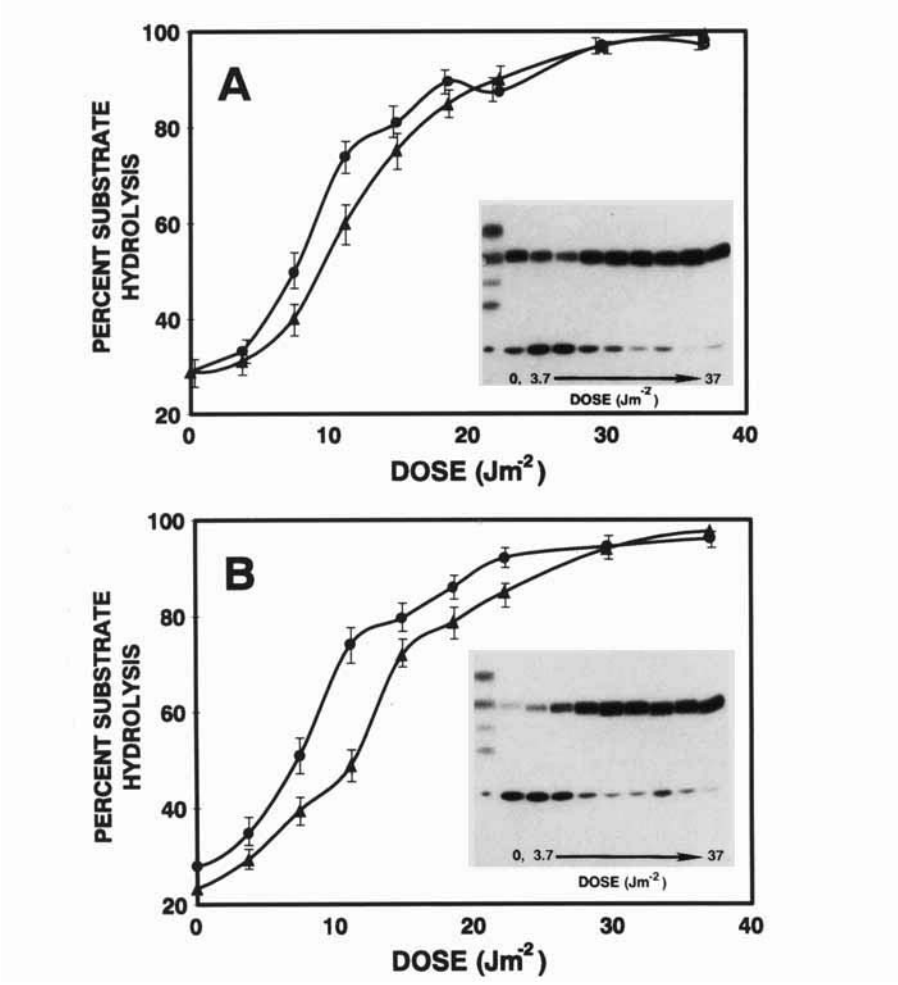

Fig. 2. Effect of UVC on cell associated peptidase activity of HeLaN70 (**A**) and HeLaS70 (**B**). Cultured cells were irradiated with 0.74 $Jm^{-2}s^{-1}$ (●) or 3.7 $Jm^{-2}s^{-1}$ (▲) flux of UVC for various times to give a total dose as indicated. Each point represents a single assay for the percent hydrolysis of P_9 at 20 h following UVC which has been normalized to account for varying cell densities. Error bars indicate the 95% C.I. which is based on the variance of 4 replicate cell counts used to determine the cell density of the particular point. Also shown are representative autoradiograph images for the results obtained with a flux rate of 0.74 $Jm^{-2}s^{-1}$ with each respective cell line. TLC plates were developed with BAW, and since the product of substrate hydrolysis was P_1, the dominant activity is aminopeptidase. From the left the fluences of UVC from the second through the tenth lanes increase from 0 Jm^{-2} to 37 Jm^{-2} in multiples of 3.7 Jm^{-2} as can be deduced from the graph; **lane 1** shows the standards [e.g., Brown et al., 1992].

ously shown that the substrate (P_9) allows for a side-by-side comparison of total vs. non-aminopeptidase activity utilizing the inhibitor bestatin. At 10 μM, bestatin inhibits aminopeptidase activity but not that of other classes of proteases [Suda et al., 1976] which we have previously verified with the peptide substrate [Brown et al., 1992]. The data of Figure 3 show the effects of UVC on both sets of activities. By increasing the frequency of sampling it is evident that the increase in the surface proteolytic activity of the HeLa cultures was bimodal with a distinct minor peak occurring at 10–15 Jm^{-2}. At fluences < 15 Jm^{-2} there was little or no difference in the level of total vs. non-aminopeptidase activities. The apparent differential increase in exopeptidase activity with doses greater than 18.5 Jm^{-2}, as well as the peak at 10–15 Jm^{-2}, was reproducible but showed some variation in magnitude between different experiments. Most importantly Figure 3B shows that for fluences < 25 Jm^{-2} there was no significant increase in permeability of the plasma membrane of the irradiated cells (trypan blue staining) which could correlate with the increase of intact culture peptidase activity, both measured 20 h after UVC. With fluences > 25 Jm^{-2} there was a dose dependent correlation between the increasing number of

TABLE II. Effects of UVC on Cell Viability and Dead Cell Numbers in Cultures at the Time of Assay for Observations in Figure 2A, B

Cultures (Flux, $Jm^{-2}s^{-1}$)	UVC fluences Jm^{-2}								
	0	3.7	7.4	11.1	14.2	18.5	22.2	29.6	37
S70 (0.74)	3[a]	—[c]	16	—	0	—	6	7	10
	[0.7][b]		[4.3]		[0]		[2.2]	[2.6]	[4.2]
S70 (3.7)	4	0	6	4	8	2	10	4	2
	[0.5]	[0]	[2.0]	[1.3]	[3.0]	[0.8]	[6.4]	[2.0]	[1.0]
N70 (0.74)	11	—	7	—	2	—	6	5	10
	[2.6]		[2.4]		[0.8]		[2.8]	[3.0]	[4.9]
N70 (3.7)	4	5	6	0	2	8	0	4	0
	[1.1]	[2.2]	[2.0]	[0]	[0.7]	[3.8]	[0]	[2.2]	[0]

[a]Actual number of dead (trypan blue staining) cells ($\times 8 \times 10^{-4}$) present in replicates of monolayers used for assay and treated similarly.
[b]Dead cells as a percentage of total cells used in assay.
[c]Not counted.

trypan blue staining cells and the steeply increasing culture peptidase activity.

As cell density influences several variables in cell culture systems, even though we have previously found no significant variation with cell density in constitutive cell surface protease activity [Brown et al., 1992], we checked to see whether this could be a modulating variable for the UVC induced effect by simultaneously comparing cultures seeded at high and low density. The data of Figure 3A show clearly that the lower density cultures have a greater protease induction such that the increase was approximately 6.5-fold in cultures with 5.10^4 cells cm^{-2} but only threefold in those with higher cell density (20×10^4 cells cm^{-2}) following 11.1 Jm^{-2} UVC.

Again cell viability changes were not a confounding variable when dead cell contamination of the assayed monolayers was evaluated. The issue, however, arises as to whether UV cell damage may potentiate the release of proteases from cells during the assay. To test this we performed dummy assays without substrate for the required incubation, collected the dummy assay medium, added substrate thereto, and then incubated for the same period (in the absence of cells) to measure any released proteolytic activity. Only trivial amounts of activity were released (Fig. 4).

Cell Density Dependence of Induced Peptidase Activity

It was previously shown that a linear relationship existed between the percent P_9 hydrolyzed and the number of intact cells assayed, usually expressed as a function of cell density [Brown et al., 1992]. However, cell density can be seen to affect the degree of UV induction of cell surface peptidase activity (Fig. 3) so a more detailed study of density effects was undertaken. The question also arose whether there was an induction of total cellular proteolytic activity or whether the increase occurred only at the cell surface.

Figure 5 represents the normalized data for the percent P_9 hydrolyzed by intact cultures as a function of increasing cell density. The percent hydrolysis of P_9 was normalized according to cell numbers to give the equivalent degree of hydrolysis that would be seen at a density of 25×10^4 cells cm^{-2}. As previously found there was no significant effect of cell density on total peptidase (− bestatin) or non-aminopeptidase (+ bestatin) activity in the sham irradiated cultures, the slight negative slope of the latter group being not significant (slope = -1.12 ± 1.12, the 95% confidence interval). However, increasing divergence of the + and − UV curves can be seen with decreasing cell density indicating that intact cultures at low cell density hydrolyze more substrate than at higher cell densities in response to UV on a per cell basis. Despite the high values of corrected peptidase activity in the low density class, the actual assayed levels were < 70% hydrolysis, the points greater than this being the result of normalization for cell numbers, with the measured hydrolysis remaining in the range of linearity for the assay.

Assays of the total cell proteolytic activities were performed on Triton-X100 cell extracts. There was no detectable perturbation in total

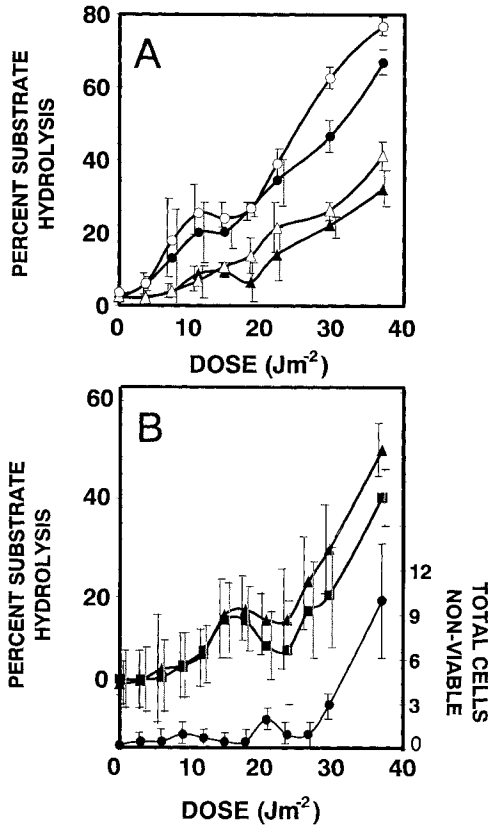

Fig. 3. Effect of varying UVC on HeLaS70. **A:** The high cell density (△, ▲) experiment was seeded with 20 × 10^4 cells and the low density (○, ●) experiment with 5 × 10^4 cells. Cultures were allowed to proceed for 56 h until they reached a density of 15.8 × 10^4 cells cm^{-2} (≈ 80% confluence) and 4 × 10^4 cells cm^{-2} (≈ 20% confluence), respectively. All cultures were then washed with HBSS and irradiated with UVC at 0.74 $Jm^{-2}s^{-1}$. Plates were irradiated in duplicate (one set for assaying and the other for cell counting) 4 wells at a time and then the cells were refed with fresh medium and further incubated for 21 h. At that time the intact cultures were assayed in duplicate in the presence (solid symbols) or absence (open symbols) of bestatin. Bars represent the 95% confidence intervals of each point. **B:** An independent high cell density experiment performed to show reproducibility; (▲) assayed without bestatin, (■) assayed with bestatin. Also shown are the total number of cells in the monolayer found to stain with trypan blue (× 4 × 10^{-4}) (●), with error bars indicating 95% of C.I.

peptidase activity in response to UVC where all peptidase activity was extracted with 0.05% Triton-X100. Assays on cell extracts were performed in a total volume of 200 μL for 2 h, but extracts were diluted to the equivalent of 1/100 the number of cells that would have been present in the intact system. Since these diluted extracts generated a degree of hydrolysis comparable to the intact cultures, the cell surface activity was approximately 1% of the whole cell protease activity capable of cleaving P_9 in an endo- or exo-fashion.

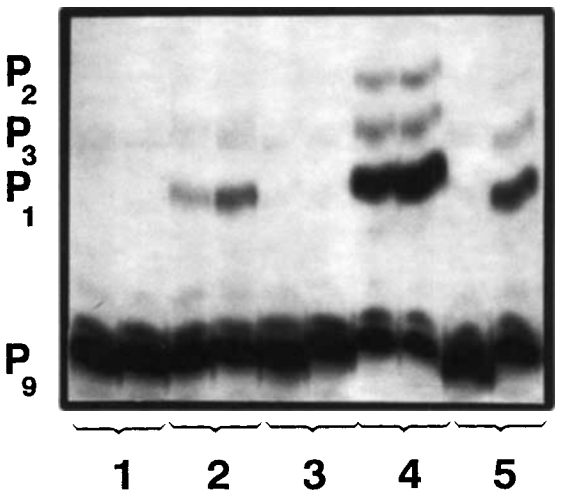

Fig. 4. Comparison of UVC response of HeLa cell surface and released peptidase activity. Duplicate HeLa S70 cultures were washed once with PBS before overlaying with 0.2 ml of PBS and incubating at 37°C for 1 h. The PBS was then removed, gently centrifuged to remove any detached cells; substrate was added and the assay (released peptidase) incubated for 1 h. The washed, intact cell layer was similarly assayed (cell surface peptidase) with another 0.2 ml of PBS/substrate for 1 h. Aliquots of assay media were immediately spotted onto TLC plates, run in BAWP, dried, and autoradiographed. The duplicate assays of released and cell surface activity are shown side by side. **Lanes 1,2:** 0 h released and cell surface, respectively. **Lanes 3,4:** 20 h post-UV. **Lane 5:** Single assays of released and cell surface peptidase 26 h post-UV.

Time Course for UVC Response

Figure 6 presents the data for the increase in cell associated peptidase activity of intact HeLa cells in response to a low dose of UVC (11.1 Jm^{-2}) with time. Intact cells, in the absence of bestatin, revealed that total surface peptidase, mainly seen as aminopeptidase activity, increased immediately after UV irradiation to reach a maximum at 16–20 h. In the presence of bestatin a lag of at least 6 h occurred before an increase in "endopeptidase" activity was observed relative to the assays of control, sham irradiated cultures. Similarly, there was a shift of 6 h in the time for which maximal peptidase activity was found (24 h). Thus, there was a differential time-course response with aminopeptidase and non-aminopeptidase activities which we have confirmed in separate experiments, although some variation in the timing and magnitude of the curves was observed.

Is Aminopeptidase Activity Responsible for the Rise in Endopeptidase Activity?

Since the aminopeptidase activity rose immediately after UVC irradiation while the endopep-

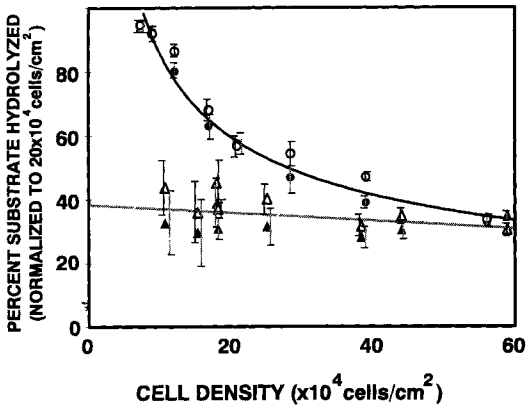

Fig. 5. Effect of cell density on the UV response of peptidase activity. HeLaS70 were cultured for 56 h at varying cell densities before sham irradiation (△, ▲) or irradiation with 11.1 Jm^{-2} (0.74 $Jm^{-2}s^{-1}$) (○, ●) followed by incubation for a further 21 h. Cell densities for each experiment were determined from 8 replicate counts, and each point is the average obtained from 2 different cultures at the same density, assayed in the absence (○, △) or presence (●, ▲) of bestatin. Results have been normalized to the percent hydrolysis of P_9 by a standardized cell density of 20 × 10^4 cells cm^{-2}, and bars represent the 95% C.I.

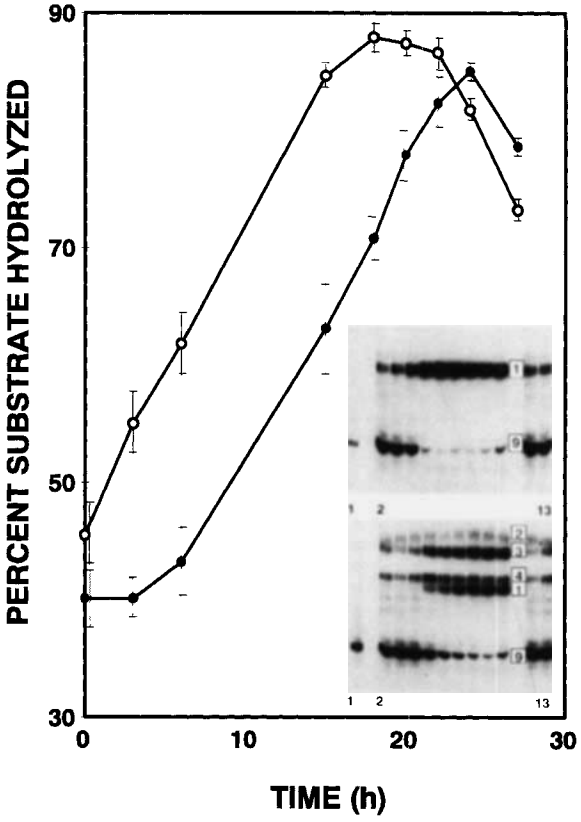

Fig. 6. Time-course induction of cell associated peptidase activity. HeLaS70 seeded at 20 × 10^4 cells and cultured in 24-well plates for 50 h and then irradiated upon reaching approximately 80% confluency with 11.1 Jm^{-2} of UVC at 0.74 $Jm^{-2}s^{-1}$ for 15 sec. Each time point assayed in the presence (●) or absence (○) of bestatin was done in duplicate on separate cultures with a minimum of 8 replicate cultures to determine the mean cell count. For each assay performed a TLC plate was spotted twice so that each datum point represents the average of 2 quantitative analyses for the same intact cell assay on 2 separate cell cultures. Error bars for the curves were calculated from the sum of the variances of the cell counts and peptidase assays and represent the 95% C.I. (Results are normalized for variations in cell densities with time.) Shown as insets are representative autoradiograph images of TLC separations of the assay media. **Lane 1:** Substrate alone. **Lanes 2–10:** Time course samples after UV irradiation up to 27 h. **Lane 11:** Peptide products (P_i) [see Brown et al., 1992]. **Lanes 12,13:** Control assays on sham irradiated cultures after 15 and 27 h, respectively.

tidase activity lagged some 6 h behind, the possibility existed that the more directly responsive exopeptidase might effect the activation of the endopeptidase(s) (e.g., by amino-terminal trimming). We, therefore, attempted to suppress endopeptidase activation, which is not inhibited by bestatin, by incubating cultures post-UVC with levels of bestatin that inhibit the aminopeptidase. First, we established that the inhibition of surface aminopeptidase by bestatin was reversible when bestatin was removed, by observing the switch in the products of P_9 hydrolysis by intact cultures from predominantly P_2, P_3, to predominantly that of the exopeptidase – P_1 (Fig. 7). The reversal, however, was not complete as can be seen by comparing Figure 7B (cells incubated without bestatin and assayed without bestatin) with Figure 7D (cells incubated with bestatin but assayed without this inhibitor) as there is more production of P_2 in the latter rather than the former. Thus residual partial suppression of the aminopeptidase allows significant accumulation of the products of the endopeptidase, at least after 12–14 h. The incomplete reversal blurs the separation of the time course of increase of exo- and endopeptidases—when measured purely by P_9 hydrolysis ± bestatin—which is normally satisfactory (Fig. 6) [Brown et al., 1992]. This complication may have contributed to the variation in time and magnitude of the responses we have observed in five separate experiments, which compared the kinetics of change in cell surface protease activity between cultures incubated with and without bestatin following UVC. Despite this variation, the data were all consistent with those graphed in Figure 8. Cultures incubated in the absence of bestatin showed the usual lag in the increase in endopeptidase activity compared with the immediate response of the aminopeptidase following UVC, whereas when cultured in the

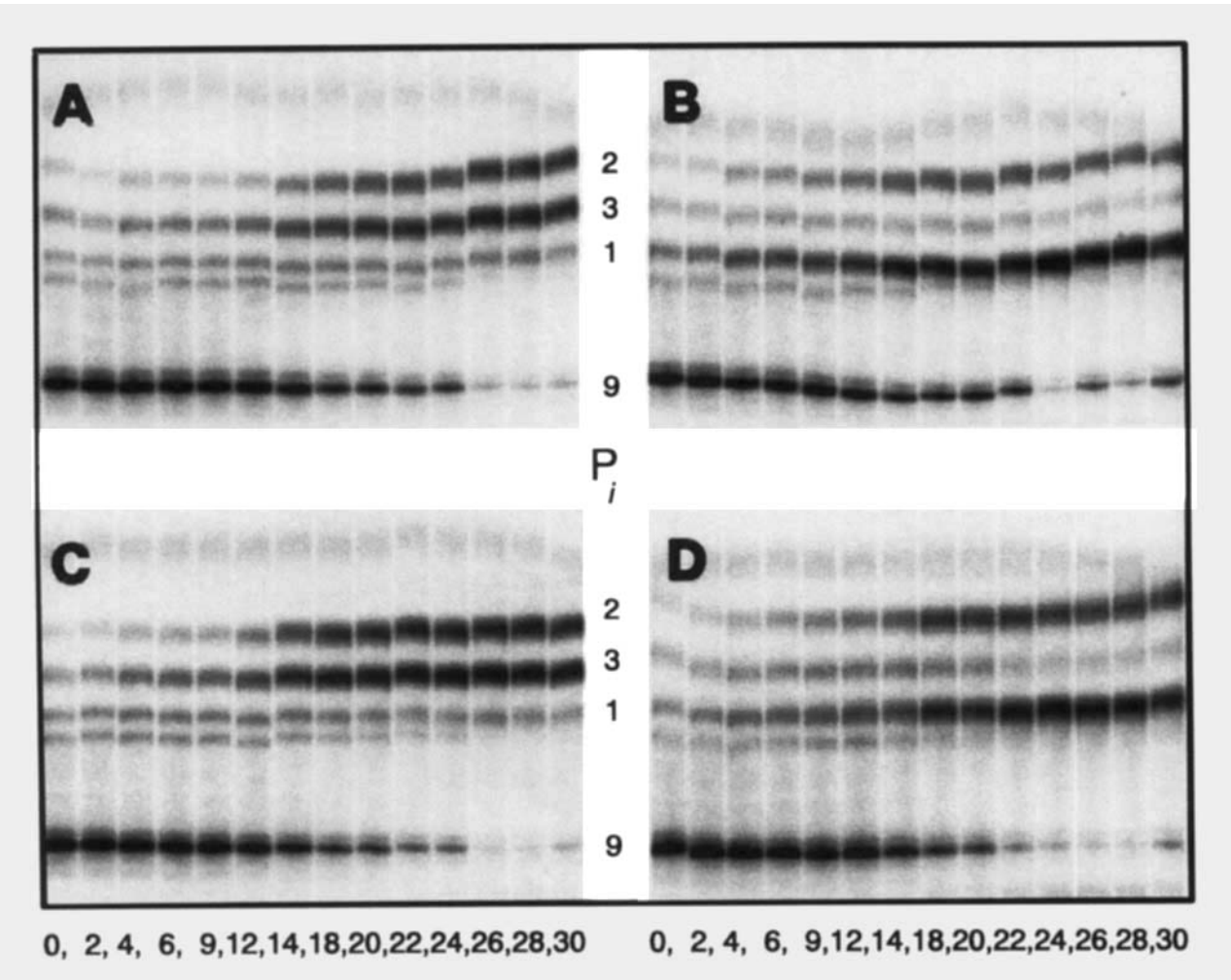

Fig. 7. Incomplete reversibility of bestatin inhibition of cell surface aminopeptidase activity of cultures incubated with bestatin (10 μM) following UVC (11 1 Jm ^{2}) irradiation Each panel is the phosphorimage of a TLC plate on which were spotted samples from the assays conducted on intact cultures at the 14 indicated times after UVC and then developed with the BAWP system The bands are identified as the substrate P_9 with products P_1, P_2, P_3, and a trace band of P_5 **A:** Cultures incubated in fresh medium without bestatin and assayed with bestatin **B:** Cultured without bestatin, assayed without bestatin **C:** Cultured with bestatin, assayed with bestatin **D:** Cultured with bestatin, assayed without bestatin

presence of bestatin the activation of both exo- and endopeptidases is delayed, and the lag between them reduced, or eliminated. A consistent finding, also, was that the maximum activity achieved occurred at the same time (20–25 h post-UVC) and exceeded the levels observed in cultures incubated without bestatin. No difference in cell viability was found between cultures incubated with or without bestatin for 25 h nor did the presence of bestatin change the surface protease activity of sham irradiated cultures. These data do not favour a role for the exopeptidase in the activation of the endopeptidase at the cell surface, but a modulating role cannot be excluded.

Is the Response Post-Translational?

Effect of cycloheximide in culture on HeLaS70 post-irradiation. We have previously shown that inhibition of protein synthesis (> 85%) with cycloheximide (10 μg/ml) did not abolish the release of TGFα from UVC irradiated cells; rather, cycloheximide itself was capable, alone, of provoking the release of this growth factor from its precursor in the plasma cell membrane of these cultures [Chenevix-Trench et al.,

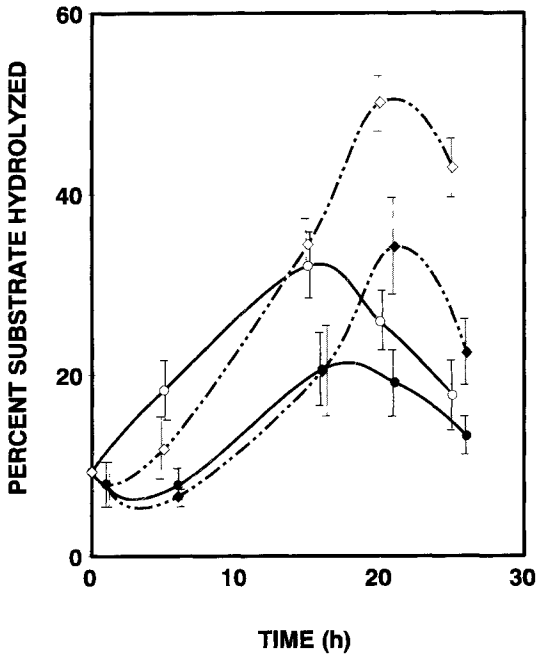

Fig. 8. Time-course induction of cell associated peptidase activity for cultures incubated in fresh medium containing bestatin (◇, ◆) or not (○, ●) following UVC. Each point represents the mean of quadruplicate assays, corrected for varying cell density, in which the associated error bars are the 95% C.I. determined from the sum of the variances of the cell counts (8 replicates) and peptidase assays. Open symbols (○, ◇) are assays in the absence of bestatin after cells were carefully but thoroughly washed in HBSS and closed symbols (●, ◆) for assays in the presence of bestatin. The results portrayed are separate experiments from those represented by Figs. 6 and 7.

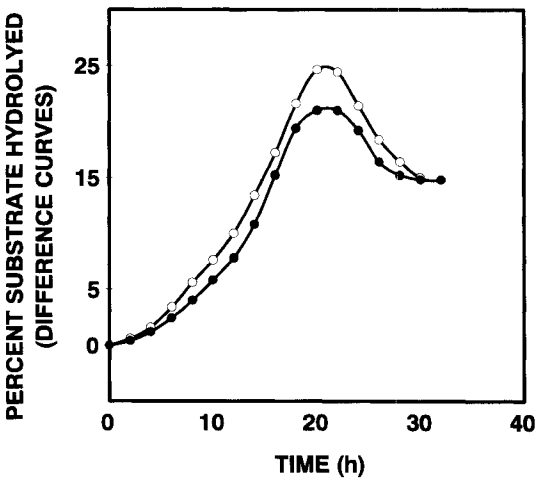

Fig. 9. Time-course effect of cycloheximide (10 μM) on unirradiated HeLa S70. Cell cultures, seeded at 20 × 10^4 cells, were allowed to proceed for 56 h until they reached a density of approximately 70% confluence before replacing the media with fresh media containing cycloheximide. Cells were then returned to the incubator until selected for assaying. The difference curves are generated from spline-fitted curves to data obtained for substrate hydrolysis of cultures assayed in the absence (○) or presence (●) of bestatin and subtracting the appropriate levels of control activity.

1992]. Cycloheximide was, therefore, trialed for its ability to modulate the post-UVC course of peptidase activity in intact HeLa cell cultures. Similar to the experience with bestatin, no two experiments yielded curves with identical time/activity profiles, but 4 separate experiments failed to show any inhibition of either aminopeptidase or endopeptidase activity. Cycloheximide added immediately after UVC had no discernible effect on the increase in aminopeptidase activity during the first 15–20 h post-UV incubation, but thereafter the levels actually exceeded those attained in its absence. Furthermore, cycloheximide abolished the lag between the two activation curves (amino- and endopeptidases) potentiating the final levels attained after 24 h (data not shown). Although cycloheximide interrupted cell growth it did not change cell viability in UV irradiated or control cultures over the time course of the experiments.

To investigate the possibility that the increase in the UVC response in the presence of cycloheximide might be due to an independent additive effect of the inhibitor, we checked the cell surface peptidases of unirradiated cultures incubated with it. When treated with cycloheximide alone, control cultures showed a significant increase in cell surface peptidase activity. As seen in Figure 9, the increases in both exo- and endopeptidases occurred almost immediately and without significant lag between the two classes of peptidase, quite unlike the response to UVC. Together their curves of activation rose to a maximum at 20–22 h, falling 40% in the next 10 h. Cycloheximide again arrested cell multiplication but did not increase the proportion of dead cells in the assayed cultures, nor did it alter profiles of the products from P_9.

DISCUSSION

The rationale for initiating these studies was the expectation that the UVC induction of TGFα in a variety of epithelial/epidermal cells including HeLa cell cultures [Ellem et al., 1988] would prove to be effected by the activation of a cell surface protease [e.g., Chenevix-Trench et al., 1992] capable of excising the mature 50 amino acid form from its membrane bound precursor [Bringman et al., 1987; Teixidó and Massagué, 1988; Pandiella and Massagué, 1991]. We have

shown that UVC at fairly low doses does, in fact, result in increases in an endopeptidase accessible to an externally supplied substrate, cognate with the cleaved sequence of proTGFα, in the time frame necessary to be responsible for the TGFα release (beginning < 12 h with maximum at 24 h [Chenevix-Trench et al., 1992; Ellem et al., 1988]. However, a number of criteria need to be satisfied before this activity can be said to be the proven regulator of proTGFα cleavage in HeLa and other cells, and these studies are being actively pursued. The present report is focussed on the broader phenomenon: the discovery that at least two classes of candidate ectopeptidases of HeLa cells are activated by low doses of UVC.

The peptidase activities we have measured in intact, viable cell cultures satisfy the principal criteria enunciated by Karnovsky [1986] for identifying an enzyme as an ectopeptidase. First, the peptide substrate is hydrolyzed by intact cells without lag and with the anticipated first order kinetics. It is unlikely that P_9 has easy access to the cell interior with immediate liberation of its products, since it has a hydrophilic serine-histidine-amide pair at the C-terminal of an otherwise hydrophobic nonapeptide. Furthermore, in cultures undergoing the exo- and endopeptidase activation with low doses ($< 25\ Jm^{-2}$) UVC, we found no evidence for increased numbers of dead (permeable) cells which might liberate intracellular peptidases thereby contributing a trivial explanation for the phenomena, nor could we detect significant accumulation of relevant peptidase activity in the medium of the cultures. Cell death could, however, explain the dose dependent increase in culture peptidase activity observed 20 h after high doses of UVC ($> 25\ Jm^{-2}$). Secondly the exo- and endopeptidase activities were not extractable from the intact cultures by 1 M NaCl, nor by 0.05% Brij 35 or Tween 20 in isotonic salt solution (data not shown). Thirdly, the different time course of the activation of the putative ectoenzymes (6 h lag between them after UVC; synchronous after cycloheximide) argues against a trivial explanation, such as loss of membrane permeability for peptidases or substrate and favours surface sites for these enzymes, with different activating triggers. When the proteases have been isolated and purified we will generate antibody probes which can be used to determine their sites in fixed cells and to follow their trafficking pathways, and UV modulations, thereto.

While our attention was directed to an explanation of the UVC effects on the status of a single growth factor and therefore a single specific protease, we have found that UVC induced changes in two classes of peptidase with different kinetics. The implications are that we are observing a global, UV induced, cellular reaction which is regulated by interactions with other cells. Thus the aminopeptidase activity, which seems to have little direct relevance to the endopeptidase activation or to TGFα excision, may well be involved in general cell behaviour such as the inflammatory reactions mediated by leukotrienes. Thus, preliminary characterization in this laboratory of the UVC activated aminopeptidase activity of HeLa cells shows it to be a Zn^{++} enzyme and leukotriene A_4 hydrolase is a Zn^{++} containing aminopeptidase [e.g., Minami et al., 1990] converting an epoxide moiety of LTA_4 to LTB_4, a potent granulocytic chemoattractant. The enzyme responsible for the N-terminal trimming of kallidin to bradykinin which mediates the vasodilator, chloride transport, and phospholipase A_2 activating characteristics of inflammation is also an aminopeptidase [e.g., Clements, 1989]. The aminopeptidase activation might also be part of a spectrum of cell surface proteases concerned with cleaving cell proteins into nonantigenic fragments to avoid the potential autoimmune complications, in vivo, of their release or exposure as a consequence of perturbed cell function and cellular stress—the "restriction proteases" proposed by Lefkovits [1986].

However, the most obvious global phenomenon of which the present cell surface peptidase activations may be a part is the MGSR codified recently [Herrlich et al., 1986] as a mammalian analogue of the bacterial "SOS" response [e.g., Walker, 1985]. A major difference between the MGSR events and the response of the UV induced ectopeptidases described here is that while these latter are post-translational activations, the various (> 14) proteins involved in the MGSR are due to AP1 driven transcriptional activation of their genes with subsequent increased translation [e.g., Kaina et al., 1989]. In the case of the UV induced ectopeptidases it may not be too farfetched to seek a mechanism by which the activation is a step in a proteolytic cascade beginning with activation of proteolysis by binding to a UV induced lesion (e.g., thymine dimer formation as in Rec A protein proteolytic activation [Phizicky and Roberts, 1981] which

is then relayed to the cell surface by trafficking or a series of proteolytic activations effecting zymogen-type cleavage of the aminopeptidases and endopeptidases. There are several reports of proteins which bind to UV irradiated mammalian DNA in situ, and which are candidates to trigger the cascade of events that follow UV damage [Glazer et al., 1989; Toschi et al., 1988]. Activation of proteolytic function in these proteins following binding to damaged DNA is clearly worth looking for.

Two proteinases—collagenase and plasminogen activator—are known to be induced by UVC [Angel et al., 1987; Miskin and Ben-Ishai, 1981], but both are secreted enzymes rather than ectoenzymes and do not have the substrate specificity to hydrolyze P_9. They could perhaps be involved in proteolytic activation of latent ectoenzymes by cleaving appropriate bonds in zymogen precursors but we have no evidence for this. The kinetics of induction of collagenase and plasminogen activator (beginning 8–10 h post-UV, plateauing at 48 h) would not seem fast enough to precede the activation of the HeLa cell surface proteases. Also, the fact that addition of cycloheximide to control cultures leads to an almost immediate and synchronous rise in both ectopeptidases would appear to eliminate such a mechanism to explain the activation following exhibition of this inhibitor, at least. Furthermore, since cycloheximide eliminates UV induction of plasminogen activator [Miskin and Reich, 1980] but stimulates the ectopeptidase response, there is no case for the involvement of the former protease in the activation of the latter enzyme(s).

An alternative explanation for the elevation of ectopeptidases following UVC might lie in the cell cycle perturbing effects of UVC. Should the ectopeptidases be expressed differentially during the cell cycle then arrest of the flow of cells through the cycle at particular phases would result in ectopeptidase activities commensurate with the average value of that phase—be it high or low. Fluences of approximately 10 Jm^{-2}, such as used in this study, and capable of inducing the peak of induced ectopeptidase activity (without cell death), cause a major accumulation of HeLa cells in S-phase over the time interval of ectopeptidase activation [Ley and Ellem, 1992; Wang et al., in preparation]. A thorough study of ectopeptidase activity on physiologically synchronized cultures will allow this mechanism to be assessed for its relevance to the present report.

The function of cell surface proteases has been the subject of speculation for many years [e.g., Kenny and Turner, 1987]. The endopeptidase activity described in this report is a candidate TGFαase since it has a similar product profile to human leukocyte elastase [Brown et al., 1992] which has been shown to be capable of cleaving TGFα from its plasma membrane-bound precursor [Mueller et al., 1990]. Its induction may explain the UVC induced release of TGFα from irradiated cells which in turn may be involved in juxtacrine, autocrine, paracrine, and even endocrine effects, in vivo. Release of other bioactive proteins from cell surfaces by modulation of endopeptidase activity must clearly be an important avenue of inquiry as part of a general mechanism for local tissue responses to variation in local conditions. Whether these or other surface proteases may also be responsible for the decrease in EGF receptor function which has been observed to follow UV irradiation [Brooks et al., 1990; Matsui et al., 1989; Ley and Ellem, 1992] needs to be determined by study of variations in the structure of that receptor.

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