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Induction of Cell Surface Peptidase Activity: A Global Response to Cell Stress Correlated with Apoptosis

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Abstract We have previously characterized the stimulation of HeLa cell surface peptidase activity directed toward a nonapeptide substrate in response to low fluences of ultraviolet irradiation [Brown et al. (1993): J Cell Biochem 51:102–115]. To explore the hypothesis that this comprised a global response to cell stress featuring the interruption of DNA synthesis, a variety of agents affecting macromolecular synthesis were applied to HeLa cell cultures. Actinomycin D, 5,6-dichloro-1β-ribofuranosyl benzimadazole, mitomycin C, ultraviolet light, and cycloheximide at doses which inhibited cell growth, but fell short of increasing the proportion of cells which had lost cell membrane impermeability to trypan blue, resulted in the concentration dependent increase in both amino- and endo-peptidase activities of intact HeLa cell cultures. γ-Irradiation, despite inhibiting an increase in cell number over a 20-h observation period, had no effect on the expressed level of cell surface peptidase activity nor did the accumulation of cells in S or G_2 phase by thymidine parasynchronization. Some of these agents were found to increase the proportion of cells in the culture undergoing apoptosis (programmed cell death), and a strong correlation was found between the extent of apoptosis and the degree of elevation in cell surface peptidase activity. Higher concentrations of perturbants in some instances increased the percentage of cells that were nonviable and an associated release of intracellular proteases overwhelmed the linear correlation with apoptotic cells. The present data do not distinguish between a homogeneous elevation of surface peptidase activity in all cells of treated cultures or the heterogeneous increase in only preapoptotic or apoptotic cells. Since sunburn of the skin increases both the occurrence of apoptotic keratinocytes (sunburn cells) in the affected epidermis and the release of membrane bound cell activators such as transforming growth factor α, it is suggested by way of extrapolation of these in vitro results, that the increase in cell surface proteolytic activity plays an integral part in the reparative responses of the epidermal cells in vivo.

Key words: apoptosis, cell surface proteases, ectopeptidases, preproTGFα, UVC-irradiation, growth factor regulation

INTRODUCTION

The release of immunologically detectable levels of transforming growth factor-α (TGF_α) from the epidermis, or cell lines derived therefrom, in response to ultraviolet (UV) irradiation has been implicated [Ellem et al., 1988; Murphy et al., 1990] in contributing to the known mitogenic effect of sunlight [Rosdahl and Szabo, 1978; Stierner et al., 1989; Pearse et al., 1987]. Coupled with somatic mutation incurred by the irradiated cell, the release of TGF_α and other growth factors may also promote neoplastic transformation and malignancies of the epidermis. Exposure to the UV component of sunlight also gives rise, however, to the histopathologic presence of the "sunburn cell" which is a manifestation of apoptosis of the epidermal keratinocyte [Weedon et al., 1979]. It is therefore quite likely that epidermal cell multiplication in response to UV is driven primarily by the need to effect tissue repair [Danno and Horio, 1987] at the cost of the unwanted appearance of dangerously mutated "precancerous" cells.

The conversion of TGF_α from a membrane-bound juxtacrine factor, capable of mediating cell–cell attachment and differentiation [Wong et al., 1989; Brachmann et al., 1989], to a diffusable mitogen in response to UV has been ascribed to a post-translational mechanism that is not reliant on de novo protein synthesis [Chenevix-Trench et al., 1993]. Indeed, the use of chemical agents that inhibited macromolecular synthesis, which included the use of cycloheximide, were themselves found to result in the significant release of TGF_α from treated cultures which

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was augmented on co-exposure to UV. As all agents effected a secondary interruption of DNA synthesis it was suggested that the release of TGF_α was part of a global response akin to the "mammalian genetic stress response" [e.g., Herrlich et al., 1986] and it followed that TGF_α may be a component of EPIF [Schorpp et al., 1984]. The exact composition of EPIF, an extracellular protein-inducing factor that is released into the conditioned media of irradiated cultures, is presently unknown though it is characterized by its ability to reproduce in unirradiated cultures the stimulatory effect of de novo synthesis of an array of proteins that are similarly induced in DNA damaged cells [Maher et al., in press].

The release of TGF_α from its membrane bound precursor ($proTGF_\alpha$) in response to UV-irradiation is presumed to occur by proteolytic processing by analogy to the stimulated release of TGF_α from CHO-transfected cells in response to phorbol ester treatment [Pandiella et al., 1991]. Whether this is effected by specific proteases or is a degenerative process is open to conjecture [Capelluti et al., 1993] but recent evidence now indicates that the signal for processing is mediated by the cytoplasmic C-terminal valine residue of the precursor [Bosenberg et al., 1992]. It is envisaged that this novel signalling and recognition pathway is activated producing a conformational change within $proTGF_\alpha$, which is transduced across the plasma membrane resulting in a higher susceptibility of the extracellular domain to cleavage by a resident protease(s). Concordant with these observations, we have recently reported the general stimulation of cell surface peptidase activities by low fluences of UV irradiation and have proposed that the endopeptidase activities identified are those of potential candidates for the processing of TGF_α [Brown et al., 1993]. We also observed that cycloheximide alone had the ability to initiate a peptidase response which was augmented by co-exposure to UV.

The combined evidence would indicate that both UV and cycloheximide can initiate reactions in HeLa cells which result in ectopeptidase activation of a global nature and may be responsible for the release of TGF_α. Since both agents result in the rapid interruption of DNA synthesis we have extended our studies herein to explore whether the response is common to a variety of agents capable of arresting cell growth. We found that induced cell surface peptidase activity occurs with many, but not all, the perturbants and strongly correlates with the extent with which apoptosis is induced in the culture monolayer rather than disturbances of the cell cycle per se.

MATERIALS AND METHODS

Materials

Chloramine T was purchased from BDH Chemicals Ltd. (Poole, England); fetal bovine serum (FBS) was obtained and processed in-house after collection of blood from a local abbatoir; RPMI-1640 growth medium, Gibco Laboratories (Grand Island, NY); trypsin, penicillin and streptomycin, Commonwealth Serum Laboratories (Melbourne, Victoria, Australia); actinomycin-D (ActD), bestatin, cycloheximide (Chx), 5,6-dichloro-1-β-ribofuranosylbenzimidazole (DRB), mitomycin-C (MitoC), pepsin, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), and Triton-X100, Sigma (St. Louis, MO); thymidine, Calbiochem-Behring (La Jolla, CA); Whatman LK6D channeled TLC plates, FSE (Brisbane, Australia). All other reagents were of AR grade. All aqueous solvents were prepared with Milli-Q purified water using in-house doubly distilled water.

Methods

Cell culture. HeLa cell cultures, free from mycoplasma contamination as judged by Hoechst 33258 staining [Chen, 1977], 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 [Brown et al., 1992]. Cells cultured for >20 passages in RPMI-1640 supplemented with 10% FBS, heat inactivated at 70°C (S70), are designated HeLaS70. Cells maintained with 56°C heat-treated serum are referred to as HeLaS56. Cultures grown in 24-well plates (Linbro, 2 cm^2) were seeded typically at 5–10 × 10^4 cells in 1 ml of media and allowed to incubate in a humidified 5% CO_2 atmosphere for 56–72 h before manipulation.

Ultraviolet irradiation of cultured cells was as described [Brown et al., 1993]. Exposure of cell cultures to various antimetabolic drugs was performed by replacing conditioned media with fresh media containing the reagent at a specified concentration. The double thymidine block employed for cell synchrony was accomplished with two successive treatments of 8 mM thymidine, each of 12-h duration, separated by a 12-h pe-

riod during which the cells were incubated in the absence of thymidine.

Cell cycle analysis of HeLa cells. HeLa cells were stained with propidium iodide in preparation of flow cytometric analysis according to the method of Williams et al. [1990] with minor modification as described by Wang et al. [1993]. Flow cytometric analyses were carried out using a FACScan, single laser, flow system (Becton Dickinson) with excitation at 488 nm again as described by Wang et al. [1993].

Quantitation of apoptosis. HeLa cells were detached from washed monolayers by treatment with Versene, fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, overnight at 4°C, and stained with hematoxylin and eosin (H&E) [Kluck et al., 1993]. The presence of early or late apoptosis was assessed from random fields and for most samples a total of 500 cells were counted with the operator unaware of the experimental status of the slides. Condensation of chromatin into sharply delineated masses, mostly in membrane-bound nuclear fragments was characteristic of the morphology of apoptosis [Kerr et al., 1972]. Toluidine-blue stained semithin sections and transmission electron microscopy (TEM) [Harmon et al., 1990] were used to verify classification as apoptosis.

Peptidase assay. The peptidase assay has been previously described [Brown et al., 1992, 1993]. Briefly, a nonapeptide substrate was synthesized to be cognate with the N-terminal cleavage site of mature TGF_{α} but contained an added N-terminal tyrosine to permit radio-iodination ($_{H}$-YVAAAVVSH-$_{NH_2}$, denoted P_9 herein). The substrate was incubated (30–120 min) at 37°C in the presence of intact monolayer cultures of HeLa cells from which growth media had been aspirated and followed by a wash with Hanks balanced saline solution (without phenol red indicator) (HBSS), leaving an intact monolayer free of detached cells either for assaying or for cell counting. Monolayers were assayed for peptidase activity in either the presence or absence of bestatin, an inhibitor of the cell associated aminopeptidase activity of intact HeLa cell cultures [Brown et al., 1992, 1993]. Assays were usually performed by adding 50 μl of a solution of P_9 substrate (approx. 250,000 cpm; 30 pmol) to cells overlayed with 200 μl of HBSS. After the appropriate incubation time (typically 60 min) a 45-μl aliquot of the assay solution (supernatant) was removed and immediately spotted onto a multichanneled TLC-plate (Whatman, LK6D) while allowing the remainder of the assay to continue for a longer period. Intact cell assays were terminated after 120 min by removal of the incubation mixture (200 μl) from the cells and subsequently freezing over dry ice before storing at −20°C to be available for repeat analysis, if required. Released (soluble) peptidase activity was determined for HeLaS70 cultures by taking washed cell monolayers and overlaying with 200 μl of HBSS for 1 h. The HBSS was then removed, gently spun for 1 min at 50*g* and to 100 μl of the supernatant, 25 μl of P_g substrate was added and assayed for 60 min at 37°C.

The substrate P_9 was separated from its products by developing the loaded TLC-plates in butanol/acetic acid/water at 100:10:30 [Brown et al., 1992]. Radioactive bands were visualized after exposure of the TLC-plate to Storage Phosphor Screen (Kodak) with the image realized by phorphorimage analysis (Molecular Dynamics, Sunnyvale, CA). Quantitation and analysis of the results was done with ImageQuant software package (Version 3.15).

RESULTS

Cell Number, Appearance, and Viability in Response to Perturbants

Since the serum used in the culture medium itself contained protease activity capable of hydrolyzing the peptide substrate used [Brown et al., 1992, 1993] it was necessary to observe whether cultures grown with serum inactivated at 70°C (HeLaS70) to remove endogenous proteolytic activity behaved differently from those grown in standard serum (HeLaS56). HeLaS56 and HeLaS70 monolayer cultures were found to respond differently to a 24-h exposure with ActD (final concentration of 3.0 μg ml^{-1} in fresh media). HeLaS56 cultures exhibited no obvious differences in morphology over the first 6 h, whereas HeLaS70 were seen to round up as early as 2 h after application. By 16 h, attachment was tenuous with a significant number of HeLaS70 cells readily displaced by agitation or aspiration of the media; by 20 h, few cells remained attached. By contrast, HeLaS56 were more resistant and, although rounded in appearance, after 20 h of continuous exposure they were still adherent. Reducing the concentration of ActD to 0.3 or 0.15 μg mL^{-1} did not decrease the rounded appearance of either cell line after a 20-h exposure but did result in increased adher-

ence of HeLaS70 cells. Cell growth was blocked at all concentrations of ActD employed, with no increase in total cell numbers seen over those at time zero. The use of ActD at 3 μg mL^{-1} did, however, result in a significant staining of cells with trypan blue (Table I). Studies by Yung et al. [1990] with HeLa cells have demonstrated that a 30-min exposure to ActD at 1 μg mL^{-1} or a 2-h exposure at 0.25 μg mL^{-1} is sufficient to induce irreversible inhibition of cell growth and RNA synthesis.

DRB, an inhibitor of nuclear, heterogeneous RNA processing to mRNA, also had a profound effect on HeLa cells when used at 93 μM, a dose known to inhibit the appearance of mRNA in the cytoplasm [Tamm and Sehgal, 1978]. After a 20-h exposure cell numbers were depressed and blisterlike protrusions (blebbing) on the surface of rounded cells were evident, with HeLaS70 cells more affected. A lower concentration of DRB (50 μM) had no obvious effect on the cells or on the inhibition of their rate of multiplication. The DNA crosslinking agent MitoC, which effectively blocked any increases in cell numbers for both cell lines, also had a significant effect on cell appearance. Although the cells showed no signs of rounding and had little detachment, they were characterized by blebbing, which was more pronounced than that seen with DRB. Brief exposure of cells to MitoC (10 μg mL^{-1}) for 30 min, before returning to fresh media and incubating for 20 h, resulted in cultures indistinguishable from controls except for the inhibition of cell multiplication (50%).

Chx, previously shown to be an effective inhibitor of HeLa cell multiplication [Brown et al., 1993; Schulte et al., 1992; and Table I] also gave rise to morphological changes in the cell population with the worst affected cells staining with trypan blue. These changes were diminished with Chx at 5 μg mL^{-1} and were almost nonexistent at 2 μg mL^{-1}, even though cell numbers remained suppressed. As with all agents, the effect of Chx was more apparent with HeLaS70 than with HeLaS56 cultures, as was the case for UVC-irradiated cells, the morphology of which has been previously described [Brown et al., 1993]. Exposure to γ-irradiation had no visible effect on either cell line.

Continuous exposure of HeLaS56 cultures to TPA at 100 ng mL^{-1} over a 20-h period had no effect on cell viability, cell multiplication, or cell appearance. By contrast, HeLaS70 cells, although quite adherent at 5–6 h, were distinctly rounded and nonadherent by 20 h, although they continued to divide. Curiously, the cells did not take up trypan blue and in contrast to the floating cells seen, for example, in response to ActD, they were not aggregated but lay as single cells on the floor of the culture well.

Peptidase Activity of Intact HeLa Cultures

The histograms of Figure 1 show the relative levels of P_9 substrate hydrolysis, corrected for cell numbers, for intact cultures of HeLaS56 and HeLaS70 determined at 6.5 h and 18 h following exposure to the indicated agents. Most notable was the finding that UVC-irradiation is

TABLE I. Effect of Perturbants on Washed HeLa Monolayers

		HeLa S56			HeLa S70		
Agent		Cell density ($\times 10^{-4}$ cells cm^{-2})	% nonviable[a]	% floating[b]	Cell density ($\times 10^{-4}$ cells cm^{-2})	% nonviable	% floating
Control		10.4 ± 1.1	5.1	0	15.6 ± 2.0	4.0	0.1
ActD	3.0 μg ml^{-1}	3.3 ± 0.4	23.1	27.8	5.2 ± 0.7	19.0	47.3
	0.3 μg ml^{-1}	—	—	—	7.1 ± 0.9	7.3	24.0
	0.15 μg ml^{-1}	5.8 ± 0.6	7.0	4.1	9.9 ± 0.8	5.6	1.4
DRB	93 μM	6.1 ± 0.6	4.7	9.0	7.6 ± 0.8	7.5	4.9
MitoC	5 μg ml^{-1}	5.4 ± 0.4	4.0	12.5	8.4 ± 0.8	6.3	5.7
TPA	100 ng ml^{-1}	10.1 ± 0.9	2.5	1.9	15.1 ± 2.1	2.5	84.0
UVC	11 Jm^{-2}	6.7 ± 0.1	2.3	4.4	10.0 ± 0.3	3.1	1.7
	19 Jm^{-2}	—	—	—	7.3 ± 0.3	4.8	3.0
γ	1 krad	7.9 ± 0.5	4.8	2.0	8.7 ± 0.4	1.8	0.1
Chx	10 μg ml^{-1}	4.8 ± 0.8	2.6	7.4	7.6 ± 0.8	2.9	3.1
	5 μg ml^{-1}	—	—	—	8.3 ± 1.7	4.9	2.0
	2 μg ml^{-1}	6.1 ± 0.7	4.6	3.1	9.1 ± 0.9	2.4	1.1

[a]Percentage of cells present in the washed monolayer used for assay, which stained with trypan blue before assaying.
[b]Percentage of cells found floating after a 1-h incubation in HBSS.

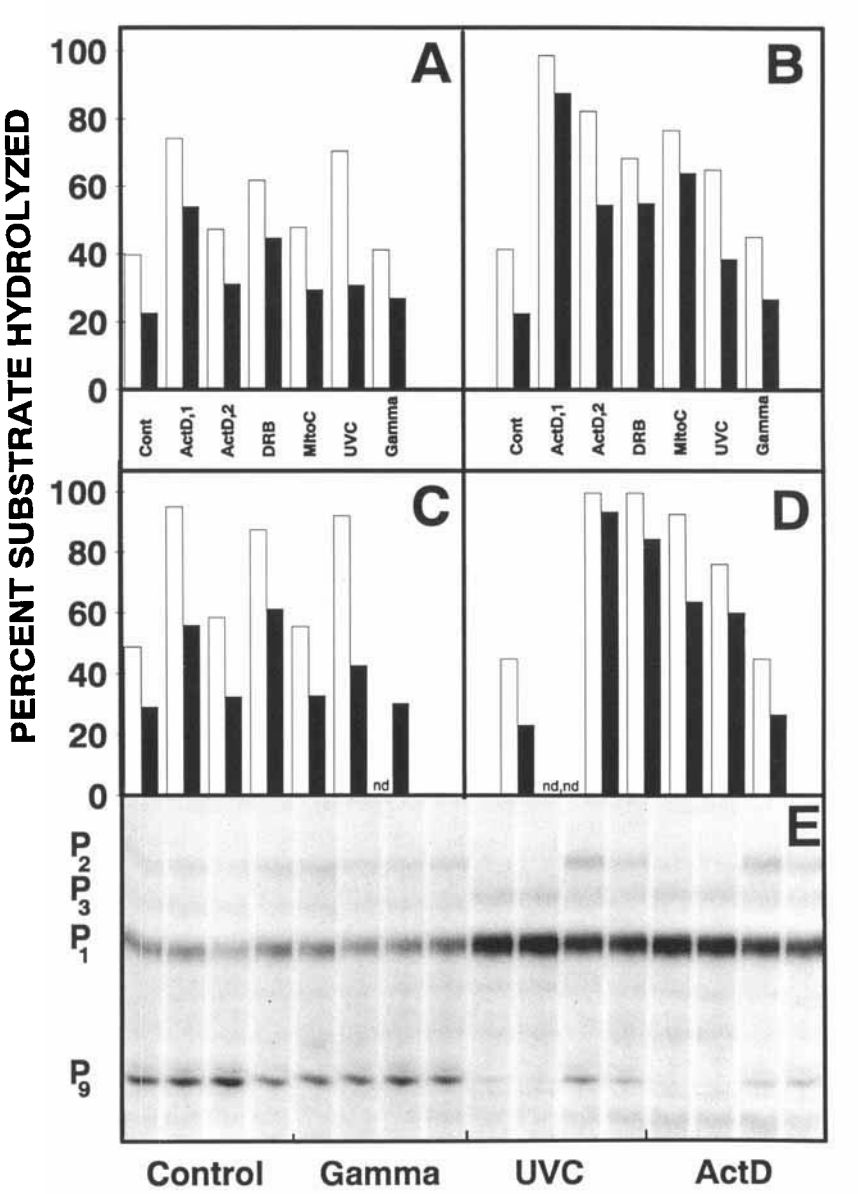

Fig. 1. Effect of antimetabolites on intact cell peptidase activity. HeLaS56 **(A,B)** and HeLaS70 **(C,D)** cultures were assayed in the absence (white bars) or presence (black bars) of bestatin after 6.5 h (A,C), by 2-h incubation with P_9, or after 18 h (B,D), by 1-h incubation with P_9, following exposure to the indicated agents; cont, control; ActD,1, 3 μg ml^{-1}, ActD,2, 0.3 μg ml^{-1}; DRB, 93 μM; MitoC, 5 μg ml^{-1}; UVC, UVC-irradiation at 11 Jm^{-2}; and Gamma, γ-irradiation at 1 krad. Only culture wells exhibiting uniform monolayers were assayed with data representing the mean of duplicate assays. Panel **E** is a separate experiment and is representative of data from which the percentage P_9 (substrate) hydrolyzed was determined in the absence of bestatin. The first pair of lanes for each set of data (Control, 1 krad γ-irradiation, 11 Jm^{-2} UVC, and 0.1 μg ml^{-1} ActD) represent TLC analysis of peptide hydrolysis by HeLaS70 monolayers (1.2×10^5 cells cm^{-2}) with the second pair representing HeLaS56. nd, not determined.

only one of several cellular perturbants to result in a time-dependent induction of HeLa cell peptidase activity. ActD, DRB, and UVC exposed cells had the greatest response with a significant increase in substrate hydrolysis observed after only 6.5 h relative to controls. Reducing the concentration of ActD to 0.3 μg mL^{-1} virtually eliminated the early response but still invoked a significant peptidase response after 18 h. The other agent that resulted in an increase in peptidase activity for intact cultures was MitoC, but only after 20 h of continuous exposure; a response at 6.5 h was not observed. Furthermore, if the cultures were incubated with MitoC at 10 μg mL^{-1} for only 30 min before returning the cells to fresh medium then no variation in pepti-

dase activity was apparent over a 20 h period (data not shown). Intriguingly, 1 krad of γ-irradiation had no effect on the expressed level of peptidase activity of intact HeLa cultures.

As was the case with the morphological differences between the two HeLa cell lines (i.e., cell rounding, blebbing, and trypan blue staining), intact HeLaS70 monolayers proved to be more responsive to a given agent when assayed for cell surface peptidase activity. This can be seen by comparing Figure 1C with 1A, and 1D with 1B, where the differences are consistent at both time intervals. The apparent difference in the ratios of ectopeptidase activity between the + and − bestatin assays of the various treatment groups after 20 h compared with 6 h (Fig. 1: B vs. A and D vs. C) is attributable to the nonlinear kinetics of the assay for substrate hydrolysis greater than 70% [Brown et al., 1992]. The quantitative differences between HeLaS56 and HeLaS70 responses can be accentuated by differences in cell density [e.g., Brown et al., 1993] but this was not the case here, where the cell densities for both experiments were identical (1×10^5 cell cm^{-2}). Data from a separate experiment in which the cell density (1.15×10^5 cells cm^{-2} vs. 1.20×10^5 cells cm^{-2}) and control levels of activity for HeLaS70 and HeLaS56 cultures were similar are shown in Figure 1E. The greater peptidase response to UVC or ActD is clearly visible for HeLaS70 monolayers from the greater amount of remaining P_9 in the HeLaS56 pairs. Figure 1E further confirms the lack of a peptidase response to γ-irradiation. Not shown are experiments, performed in triplicate, which revealed that TPA had no effect on intact cell peptidase activity within the duration of these experiments. Also not shown was the absence of a significant peptidase response by UVC-irradiated HeLaS70 monolayers that were cultured in Linbro 24-well plates that had supported a previous confluent HeLaS56 monolayer before being removed by trypsin treatment and a PBS wash.

Induced Peptidase Activity Is Not Due to Perturbation of the Cell Cycle

We had previously seen with UVC-irradiated HeLaS56 cells an effective synchronization of cultures where the average percentage of cells in S-phase dramatically rose from 35–45% to 90% by 16 h [Wang et al., 1993]. The possibility existed that a redistribution of the cell population with regard to the cell cycle was responsible for the UVC-induced peptidase response. The double thymidine block of Stein and Borun [1972] was therefore used to synchronize HeLa cultures without the toxic side-effects associated with the above mentioned agents.

Figure 2 compares flow cytometric analyses of propidium iodide stained control cultures with the redistribution of the cell population of HeLa cultures 3 h and 6 h after release from a double thymidine block. Again, the effect on HeLaS70 is more pronounced with a large proportion of both cell lines seen to accumulate in late S-phase (Fig. 2B,F), a situation not dissimilar to that following UVC-irradiation at 11 Jm^{-2}. However, intact cell peptidase assays performed at times corresponding to those of Figure 2 (where the cell population in S + G_2 varied from 30% to 90%) exhibited no significant variation in the level of substrate hydrolysis for either HeLaS56 or HeLaS70 cultures (all values fell within the 98% confidence interval of the mean of quadru-

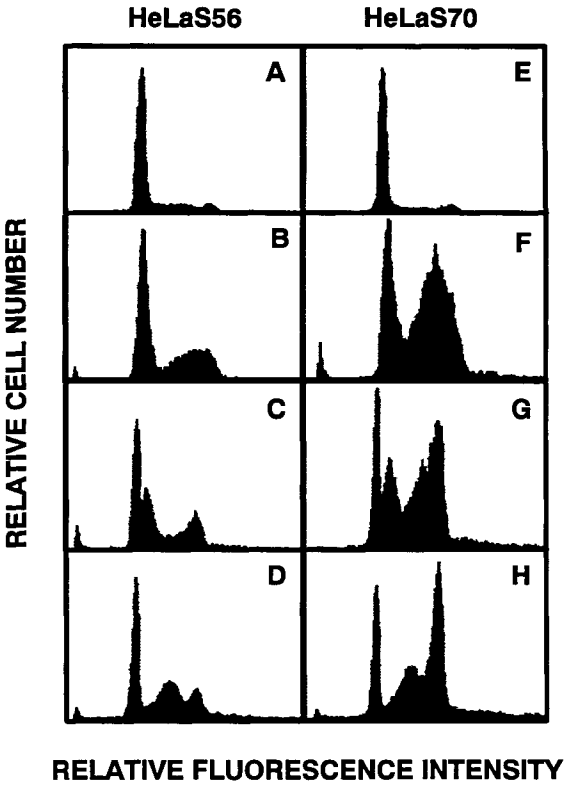

Fig. 2. FACS analysis of HeLa cells released from a double thymidine block. Histograms represent propidium iodide stained nuclei of HeLaS56 (**A–D**) and HeLaS70 (**E–H**) cultures (see Methods) released from the second thymidine block and collected at 0 h (B,F), 3 h (C,G), and 6 h (D,H). Control cultures (A,E) were exposed to the first application of thymidine but not the second and therefore represent a 24-h release from a single 12-h exposure to 8 mM thymidine.

plicate assays, performed in the presence or absence of bestatin). Thus, there was no evidence for an elevation or induction of surface protease activity of cultures enriched for cells traversing the S or S + G_2 compartments of the cell cycle.

Lost Membrane Integrity: A Confounding Variable in the Intact Cell Assay

We have previously argued that the induced peptidase activity of HeLa cells in response to UVC-irradiation (< 15 Jm^{-2}) was a surface phenomenon. This was favoured by the absence of increased numbers of adherent cells in the monolayer which stained with trypan blue and the failure to detect significant release of soluble peptidase activity into the HBSS supernatant during the course of an intact cell assay in response to UVC, compared with control cultures [Brown et al., 1993]. Further support was drawn from the differential time-course response between endo- and exopeptidase activities (assayed with and without bestatin respectively) [Brown et al., 1993]. However, incubation of HeLa cultures with ActD at 3 μg mL^{-1} was followed by significant shedding of "adherent" cells during incubation in HBSS. Considering that surface peptidase activity of cells in the intact monolayer amounts to about 1% of the total cellular peptidase activity (intra- and ectocellular) capable of hydrolyzing the P_9 substrate [Brown et al., 1993], any loss in membrane integrity of the cells shed during an assay would complicate the interpretation of an intact cell assay.

Table I confirms that cells were shed from the treated monolayers during a 1-h incubation in HBSS and that shedding was especially significant for cultures treated with ActD (3 μg mL^{-1}), DRB and MitoC-the same agents responsible for the more pronounced increases in intact cell peptidase activities. The majority of these shed cells stained with trypan blue, with the exception of HeLaS70 cultures treated with TPA (data not presented). The correlation between increased trypan blue staining cells of the monolayer and protease activity was not complete in that HeLa cultures treated with ActD (0.15 μg mL^{-1}) typically resulted in a level of shed cells comparable to UVC-irradiated cells (11 Jm^{-2}, Table I) yet had a greater peptidase response. To address the possibility that the increased "surface" peptidase activity might be entirely due to intracellular proteases released from a small proportion of "nonintact" attached or floating cells, we determined the level of protease activity liberated into the assay medium by using the supernatant from cultures incubated for 1 h in HBSS in parallel with intact cell assays.

Released Peptidase Activity Does Not Account for Total Induced Activity

HeLaS70 cultures treated with a variety of agents were assayed for total intact cell peptidase activity and for released soluble activity. For simplicity, results have been presented as histograms (Fig. 3) where the fold-increase in the intact cell peptidase assay is represented by

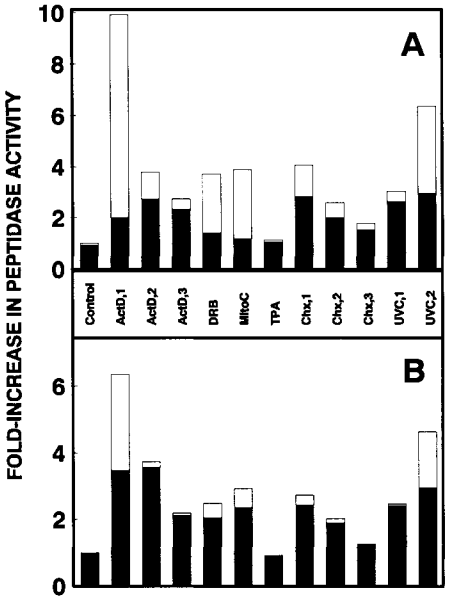

Fig. 3. Level of soluble enzyme released into the supernatant during the course of an intact cell assay. HeLaS70 cells subjected for 18 h to control conditions; ActD,1 (3.0 μg ml^{-1}); ActD,2 (0.3 μg ml^{-1}); ActD,3 (0.15 μg ml^{-1}); DRB (93 μM); MitoC (5 μg ml^{-1}); TPA (100 ng ml^{-1}); Chx,1 (10 μg ml^{-1}); Chx,2 (5 μg ml^{-1}); Chx,3 (2 μg ml^{-1}); UVC,1 (11 Jm^{-2}); and UVC,2 (19 Jm^{-2}). The cultures were then gently but thoroughly washed free of media and antimetabolite before overlaying with 200 μl of HBSS for 1 h at 37°C. The supernatant was removed, gently spun and 100 μl was removed and assayed for peptidase activity after the addition of 25 μl of P_9 substrate (250,000 CPM/assay) for 1 h in the absence (**A**) or presence (**B**) of bestatin. The intact washed cultures were assayed upon the addition of 50 μL of substrate to cells overlayed with 200 μl of HBSS and assayed for 1 h in the absence (A) or presence (B) of bestatin. The total height of the histograms represents the fold-increase in total peptidase activity, relative to control conditions, observed for an intact cell assay, while the white component represents the activity of soluble enzyme found in the HBSS supernatant. The remaining black component presumably reflects the ectopeptidase activity of the intact cells.

the total height of the bar relative to control hydrolysis. The solid black component of each histogram represents the fraction of activity remaining after subtracting out that activity which was directly attributable to enzyme released during the course of an intact cell assay measured in the HBSS supernatant. The values subtracted (the white component of each histogram) are a significant overestimation of the time average release of activity, since the soluble peptidase component was assessed with a 1-h assay after a 1-h exposure of the cells to HBSS. With the exception of TPA, as already noted, all agents were still seen to result in an induction of cell associated peptidase activity. This was so whether the cells were assayed in the presence or absence of bestatin although there is some indication of a preferential release of amino- rather than nonamino-peptidase activity.

A Correlation Exists With Apoptosis

The release of trypan blue staining cells from the washed monolayer (Table I) at levels greater than that seen for control cultures was indicative of cell death. Furthermore, cells exhibiting an apparent breakdown in intercellular (desmosomal) contacts with surface blebbing, cell rounding, and detachment was also indicative of cell death by apoptosis [Bursch et al., 1990]. Apoptosis, which manifests as condensation of both nucleus and cytoplasm [Kerr et al., 1972] is further characterized by the ability of the dying cell to exclude nonvital dyes (e.g., trypan blue) and maintain plasma membrane integrity in the early stages. Advanced stages, in vitro, will result in "secondary necrosis" as membrane lysis occurs.

By analyzing H&E-stained fixed cells of the washed monolayer (see Methods), we explored the possibility that induced peptidase activity may correlate with conditions presaging cell death by either apoptosis or necrosis. The percentage of cells in the HeLa monolayer scored as being apoptotic or necrotic following exposure to the various perturbants are reported in Table II. With the exception of TPA and γ-irradiation, all agents were seen to result in substantial levels of apoptosis, with HeLaS70 the more sensitive of the two cell lines. The sensitivity of HeLaS70 to UVC-induced apoptosis was, however, dramatically reduced if the HeLaS70 cells were subcultured on the extracellular matrix laid down by a previously existing confluent monolayer of HeLaS56 cells (Table II, footnote[d]). Necrosis, occurring by primary mechanisms, was not found to be significant, except in the case of ActD treatment (3.0 $\mu g\ mL^{-1}$).

The results suggested that those agents giving rise to the more prominent peptidase response were also responsible for greater cell death by apoptosis. Therefore the results of an experiment in which the peptidase activity and the proportion of cells in apoptosis in response to a given agent are plotted in Figure 4A. Agents are ranked from the left in the order of increased intact cell peptidase activity, which was the same for + or − bestatin. The similar trends in change of both data sets was striking whereon regression analysis (Fig. 4B) yielded strong correlation coefficients of 0.96 and 0.89 for "amino-" and "endo-"peptidase activities respectively against log[%apoptosis]. In both instances, the likelihood that this correlation occurred by chance is < 0.001.

DISCUSSION

The primary focus of these studies was to explore the possibility that the stimulation of HeLa cell surface peptidase activity, in response to UVC-irradiation [Brown et al., 1993], was part of a global response to cell stress as typified by the mammalian genetic stress response (MGSR) [Herrlich et al., 1986]. The MGSR is characterized by the upregulation of the expression of a number of genes (DNA damage-induc-

TABLE II. Percentage Apoptotic Cells[a]

		HeLaS56		HeLaS70	
Agent		A[b]	B	A	B
Control		2 (0)[c]	1 (2)	2 (1)	3 (1)
Act D	3.0 $\mu g\ ml^{-1}$	71 (0)	84 (4)	65 (6)	—
	0.3 $\mu g\ ml^{-1}$	—	15 (2)	—	—
	0.15 $\mu g\ ml^{-1}$	10 (3)	—	31 (1)	28 (3)
DRB	93 μM	30 (0)	10 (0)	39 (3)	—
MitoC	5 $\mu g\ ml^{-1}$	58 (0)	34 (1)	69 (0)	—
TPA	100 $ng\ ml^{-1}$	1 (1)	1 (1)	4 (0)	2 (0)
Chx	10 $\mu g\ ml^{-1}$	5 (1)	17 (5)	30 (1)	—
	2 $\mu g\ ml^{-1}$	5 (1)	4 (3)	11 (3)	—
UVC	11.1 Jm^{-2}	6 (1)	4 (3)	13 (0)[2][d]	13 (6)
γ	1 1 krad	2 (0)	0 (1)	2 (2)	—

[a]Percentage of cells in the washed monolayer found to be apoptotic after random counts of usually 500 cells per sample.
[b]Experiment A can be directly compared with Table I.
[c]Percentage of cells scored as necrotic.
[d]Percentage of UVC-irradiated cells found to be apoptotic when cultured in wells that had supported prior HeLaS56 monolayers after removal by trypsin treatment and a PBS wash.

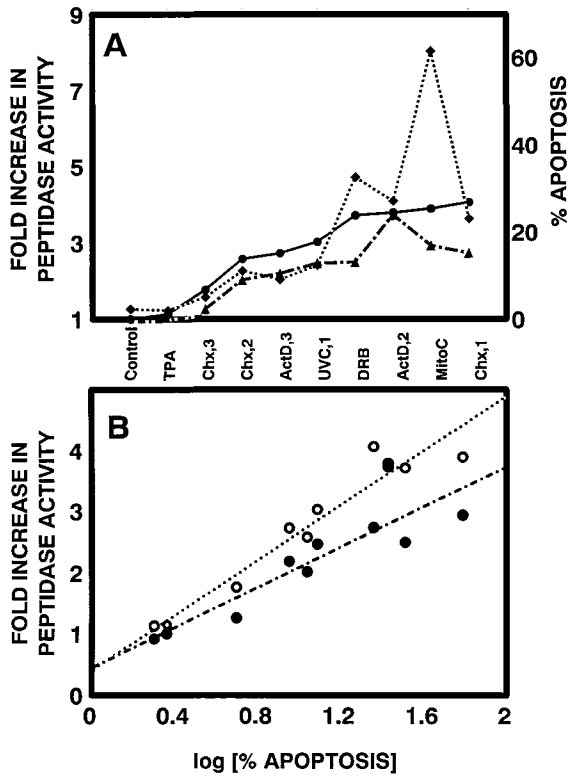

Fig. 4. Correlation between apoptosis and peptidase activity (intact cell assay) for HeLaS70 cultures. **A:** Conditions resulting in increased peptidase activity for intact cells assayed in the absence (◆...◆) or presence (▲...▲) of bestatin are plotted together with the corresponding percentage of cells in the monolayer that were apoptotic (●—●). The data are ranked from left to right in order of conditions resulting in increasing percentage of cells as apoptotic. **B:** Relationship between apoptosis and peptidase activity (intact cell assay) for HeLaS70 cultures. The fold increase in peptidase activity was plotted against the logarithm for the corresponding percent cells found to be apoptotic in the cell culture. Open circles represent data for those assays performed in the absence of bestatin (○—○), whereas the solid symbols are representative of assays performed in its presence (●...●). Linear regression analysis yielded correlation coefficients of 0.96 and 0.89, respectively.

ible genes) whose products may be involved in detoxification and repair following exposure to genotoxic agents.

The present studies have shown that exposure of HeLa cell cultures to agents that typically can induce MGSR and/or inhibit macromolecular synthesis, affected cell morphology, growth, viability and attachment. These perturbations to the cell could be correlated with the development of cell death by apoptosis within a subpopulation of the cells, the level of apoptosis correlating with the degree of induced peptidase activity of intact cultures. However, apoptotic cell counts could not differentiate between membrane intact (early) or membrane permeable (late) stages, so that the contribution of intracellular peptidases from lysed apoptotic cells to the total activity was a potentially confounding factor. Estimation of soluble peptidase contribution by measurement of released peptidase activity and of membrane permeability (nonviable, trypan blue staining cells) indicated that an increase in ectopeptidase activity remained independent of cell lysis. The possibility that any variation in peptidase activity was due to growth arrest at various points of the cell cycle was also excluded. The increases in peptidase activity would appear to be independent of transcription since it was not prevented by cycloheximide, and in this regard differed from most of the other reactions subsumed under the description of the MGSR. The SOS response in UV-irradiated bacteria provides the precedent for protease activation occurring prior to and independent of transcriptional activation [e.g., Phizicky and Roberts, 1981]. We are thus presented with the fascinating phenomenon that intact cell surface protease activation may be a marker for apoptosis in vitro and that it may subserve a physiological function in vivo.

While the homogeneity of the cell response to MGSR induction has not been reported, the present finding that the magnitude of a protease response is correlated with the proportion of cells undergoing apoptosis raises a number of issues: (1) whether the MGSR is a global response of all cells or is the property of a subset of the exposed population; (2) whether the elevation in protease activity of intact cells is a component of the MGSR or is, in contrast, a failure by the affected cells to invoke an MGSR and hence repair. There is also the question of whether the release of TGF_α from HeLa cells seen in response to many of the employed agents [Chenevix-Trench et al., 1993] is also a by-product of apoptosis. This has a clear implication as to the origin of EPIF [Schorpp et al., 1984] and UVIS [Rotem et al., 1987] release and may extend also to the observed release of IL-1α and bFGF by irradiated cultures [for review see Ellem et al., 1990]. The maturation and release of IL-1α and IL-1β has been shown to arise from peritoneal exudate cells induced to undergo apoptosis in response to a variety of agents [Hogquist et al., 1991].

Our interest in cell surface proteases stemmed from the observation that exposure of human skin to erythemal equivalent doses of sunlight

resulted in the increased passing of soluble TGF_α in the urine (Ellem et al., submitted). In vitro modelling with UV irradiation of cell lines derived from the epidermis indicated that the release of TGF_α from its membrane bound precursor was post-transcriptional and probably dependent on proteolytic processing [Chenevix-Trench et al., 1993]. Keratinocytes, which are the predominant cell in the epidermis, express the membrane anchored form of TGF_α [Gottlieb et al., 1988] but have the ability to upregulate its expression in response to exposure to its soluble counterpart or epidermal growth factor (EGF) [Coffey et al., 1987]. This has been invoked as a mechanism by which a local concentration of factor is attained for the mitogenesis of both melanocytes and keratinocytes in a paracrine reciprocating fashion [Ellem et al., 1990]. The recent findings that membrane anchored pro-TGF_α can also interact with its receptor (EGFR) on adjacent cells [e.g., Massagué et al., 1990] to mediate cell–cell adhesion as well as cell-cell stimulation [Anklesaria et al., 1990; Brachmann et al., 1989; Wong et al., 1989] suggests that it may have a major role in the signalling events between contiguous cells and may thus function to serve as a mechanism for "social control" [Raff, 1992] and maintenance of epidermal architecture [Mann et al., 1993; Luetteke et al., 1993].

Exposure of skin to excess sunlight is characterized by the presence of sunburn cells (SC) [e.g., Olson et al., 1974], which are distributed randomly throughout the affected epidermis [Daniels et al., 1961] and are caused by the solar content of UVB. These damaged keratinocytes, whose abundance is dependent on total sunlight exposure [Danno and Horio, 1987], display the morphological features of apoptosis. It is therefore a distinct possibility that in cells undergoing apoptosis the plasma membrane is enriched for proteases which may initiate the breakdown of those proteins which mediate cell-cell communication. It is also a possibility that the apoptotic cell may cleave proTGF_α, thereby converting it from one active form to another [Ehlers and Riordan, 1991]. Excess released TGF_α may then enter the circulatory system to act as an endocrine mitogen before being cleared by the renal system (Ellem et al., submitted).

The effect of supplementing growth media with FBS, heat inactivated for 1 h at 70°C, as opposed to the usual treatment of 56°C for 30 min, had a major effect on HeLa cell cultures. Most notable was the greater sensitivity of HeLaS70 cultures to undergo apoptosis in response to the presence of agents known to affect cell growth. We speculate that this reflects a greater stress load on HeLaS70 due to the inactivation or depletion of factor(s) in the 70°C inactivated serum. Possible candidate molecules are those involved in cell adhesion (e.g., fibronectin) for which evidence is provided by (1) the poor adherence of HeLaS70 to culture surfaces when initially subcultured with medium supplemented with 70°C inactivated serum [Brown et al., 1992]; (2) the relative ease with which HeLaS70 cells are displaced by trypsin treatment from culture surfaces; and (3) a differential UV effect (decreased apoptotic effect) for HeLaS70 cells subcultured on to an extracellular matrix put down by a prior HeLaS56 monolayer.

This evidence shows that a variety of perturbants cause an elevation of cell surface peptidase activity by a nontranscriptional mechanism, which may occur in all cells exposed, or may be confined to the subset of cells undergoing apoptosis. If present in all cells, it would represent a global response which may be a part of the MGSR, one default outcome of which may be apoptosis. If the peptidase activation is confined to pre-apoptotic and apoptotic cells, however, the MGSR default option may be an important element in driving the longer term function of tissue repair by release of bioactive cell surface proteins [Ehlers and Riordan, 1991]. Methods to distinguish the source of the activity within UV-irradiated populations are being explored to clarify this important question.

Our attention has been focused on TGF_α (only one of many proteins released) because of its known release from, and presence in, epidermal cells and for which a case has been made for a role in maintaining the architecture of this tissue [Mann et al., 1993; Luetteke et al., 1993]. UV-induced cleavage of mature TGF_α from its prepro- form by cell surface proteolysis may indicate reciprocal paracrine stimulation of all cell types in the epidermis to undergo cell division and movement. However, by interrupting the juxtacrine component of the TGF_α-EGFR interaction, it may contribute to the tissue repair process. The default option of apoptosis can be interpreted as part of the "fail-safe" mechanism to ensure the eradication of cells unable to clear UV-induced DNA damage before enforced cell replication might ensure the presence of a

heavy mutational load in their descendents, thereby increasing the probability of clonal neoplastic change. The role of protease released surface proteins in the functional definition of EPIF as inducer of the transcriptional phase of MGSR is clearly an important item for further study.

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