The regulation of proteolysis in normal fibroblasts as they approach confluence

Evidence for the participation of the lysosomal system

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The effect of the lysosomotropic agent NH_4Cl and the proteinase inhibitors leupeptin, Z-Phe-Ala- CHN_2 (benzyloxycarbonylphenylalanylalanyldiazomethane) and pepstatin on the degradation of intracellular proteins in Swiss 3T3 mouse and normal human fibroblasts in both the exponential and stationary (confluent) growth phases in nutritionally complete conditions was investigated. Inhibitory effects of all four agents on degradation in both growth states were detected. The increase in proteolysis normally occurring as cells approach confluence could be completely blocked by NH_4Cl , by Z-Phe-Ala- CHN_2 , or by pepstatin in the presence of leupeptin. These results suggest that the lysosomal system is responsible for the regulation of proteolysis at confluence and further confirm its role in 'basal' proteolysis in growing cells.

We have shown previously that degradation of stable intracellular proteins increases in several normal cell lines as they approach confluence and cell division slows (Dean & Cockle, 1981; Cockle & Dean, 1982). Protein degradation is also regulated by hormones and nutrients (as reviewed by Ballard, 1978). It is now clear that there are both cytosolic and lysosomal sites of endogenous protein degradation (Goldberg & St. John, 1976; Seglen *et al.*, 1981), although their relative importance varies in different nutritional conditions. In nutritional depletion lysosomal roles are substantial (Dean, 1975; Amenta *et al.*, 1977), but lysosomes also seem to play a slighter role in 'basal' proteolysis in nutritionally complete conditions (Poole *et al.*, 1978; Dean, 1979).

This present paper provides evidence, by the use of lysosomotropic agent and proteinase inhibitors, for the participation of the lysosomal system in the regulation of degradation in normal fibroblasts as they approach confluence under nutritionally complete conditions. It also confirms their participation in 'basal' proteolysis.

Materials and methods

L-[1- ^{14}C]Leucine (55 mCi/mmol) was obtained from The Radiochemical Centre (Amersham,

Abbreviation used: Z-Phe-Ala- CHN_2 , benzyloxycarbonylphenylalanylalanyldiazomethane.

Bucks., U.K.). Swiss 3T3 fibroblasts from mice and tissue-culture reagents were obtained from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.), except leucine-free Minimal Essential Medium with Earle's salts, which was supplied by Gibco Europe (Uxbridge, Middx., U.K.) Scintillator 229 was supplied by Packard Instruments Co. (Caversham, Berks., U.K.) and leucine and bovine serum albumin were from Sigma Chemical Co. (Poole, Dorset, U.K.). NH_4Cl and general laboratory chemicals, of analytical quality, were obtained from BDH (London, U.K.) Pepstatin and leupeptin were obtained from the Protein Research Foundation (Osaka, Japan). Z-Phe-Ala- CHN_2 was kindly given by Dr. Elliot Shaw, Brookhaven National Laboratory, NY, U.S.A. Pepstatin and Z-Phe-Ala- CHN_2 were dissolved in dimethyl sulphoxide and diluted into media such that the final concentration of dimethyl sulphoxide was 2%. At this concentration, dimethyl sulphoxide was not toxic and had no observable effect on protein degradation.

Cells were grown in minimal essential medium with Earle's salts (MEM) buffered with NaHCO_3 and containing 10% (v/v) heat-inactivated foetal calf serum, penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) at 37°C in an incubator gassed with 5% CO_2 in air. Swiss 3T3 fibroblasts were used for experiments at passages up to 145, and normal human fibroblasts were used between passages 10 and 20.

Growth curves were established for each cell line by cell counting, and on the basis of these, for all degradation experiments cells were seeded in tissue-culture tubes at 10^4 and 10^5 cells per tube for the exponential and stationary (where there is no net increase in cell number) growth phases respectively. DNA synthesis measured by thymidine incorporation in the confluent cultures was less than 10% of that in exponential conditions. Cells in these defined conditions were labelled for 16 h in leucine-free MEM containing 10% heat-inactivated foetal-calf serum, antibiotics and [^{14}C]leucine ($0.1 \mu\text{Ci/ml}$). After labelling, the cells were washed (twice) with 3.0 ml of MEM containing 10 mM-leucine in order to free the intracellular pools of radioactive leucine. Degradation was allowed to proceed for 24 h, in 0.5 ml of MEM containing 10% heat-inactivated foetal-calf serum, antibiotics and 10 mM-leucine to prevent reincorporation of the released label. Medium and cell fractions were precipitated with trichloroacetic acid (final concn. 5%, v/v), after adding 0.1 ml of 10% (w/v) albumin as carrier to the cell fractions, and then centrifuged at 3000 g for 15 min. All the acid-soluble fraction from the medium and half that from the cells were dispensed into scintillation vials containing Scintillator 229. The trichloroacetic acid precipitates were dissolved in 90% (v/v) formic acid and the total volume was used for scintillation counting. A channels-ratio method was used for quench correction. Degradation was expressed as:

$$100\% \times \frac{(\text{acid-soluble d.p.m. in cells and medium})}{(\text{total d.p.m. in culture})}$$

The release of acid-precipitable material into the medium was used as the upper estimate of the proportion of cells lysed during the experiment and was always less than 10%. Results are expressed as means $\pm$ S.D., where $n = 4$, and are representative of several similar experiments.

In most experiments, inhibitors of degradation were supplied only during the degradation period defined above, but with pepstatin (which was ineffective when supplied solely in the degradation phase), and with pepstatin and leupeptin together, experiments were also conducted with cells pre-incubated in the presence of inhibitors for 72 h before the degradation period.

Results and discussion

NH_4Cl has a marked inhibitory effect on the degradation in Swiss 3T3 and normal human fibroblasts (Fig. 1) which is greater in confluent than in exponentially growing cultures. At a concentration of 20 mM- NH_4Cl , a complete block of the increase in proteolysis normally occurring in cells approaching confluence is observed in both cell

types (Fig. 1). Ammonia and several organic amines, such as methylamine, propylamine and chloroquine, are lysosomotropic in that they accumulate inside lysosomes, causing vacuolation (Ohkuma & Poole, 1981) and an increase in the pH (Poole & Ohkuma, 1981); the lysosome seems to be the target for their inhibition of protein degradation (Seglen & Reith, 1976). Thus the ability of NH_4Cl to inhibit completely the increase in proteolysis in confluent cultures suggests that the lysosomal system is responsible for the regulation, although cytosolic effects of the amine cannot be completely excluded.

To substantiate further this important lysosomal role in the accelerated proteolysis at confluence, we have studied the effects of pepstatin, the group-specific aspartyl-proteinase inhibitor, which is

Fig. 1. Effect of NH_4Cl on protein degradation in (a) normal human and (b) Swiss 3T3 fibroblasts. Cells were seeded (●, confluent; ▲, exponential growth), labelled, and degradation was measured as described in the Materials and methods section. NH_4Cl was present only in the degradation medium. Points represent the means of four values ($\pm$ S.D. where greater than the span of the points).

believed to enter lysosomes and to inhibit lysosomal cathepsin D in living cells (Dean, 1979). It is useful in this regard because it seems unable to permeate cell membranes (Dean, 1975), and thus enters the cells by pinocytosis (Dean, 1979; Shaw & Dean, 1980), and is apparently sequestered by lysosomes and excluded from the cytosol. Pepstatin presented alone only in the degradation medium to 3T3 and normal human fibroblasts has no significant effect on degradation, presumably because insufficient inhibitor can be internalized within the 24 h degradation time period. In contrast, inhibition (20%)

of macrophage protein degradation has been observed in 24 h with pepstatin (Dean, 1979); however, fluid-phase pinocytosis of horseradish peroxidase is 7 times faster in mouse peritoneal macrophages than in fibroblasts (L-cells) (Steinman *et al.*, 1976; reviewed by Pratten *et al.*, 1980), which may explain this difference. Consistent with this idea was the observation that pepstatin nevertheless increased the inhibitory effect of leupeptin (see below) in 3T3 cells when present with it during the degradation

Fig. 2. Effect of pepstatin on protein degradation in (a) normal human and (b) Swiss 3T3 fibroblasts. Cells were preincubated with the inhibitors for 72 h. They were then trypsin treated, and seeded (●, confluent; ▲, exponential growth) into tissue-culture tubes, and labelling and degradation were performed in the presence of the inhibitor as described in the Materials and methods section. Points represent the means of four values ($\pm$ s.d. where greater than the span of the points).

Fig. 3. Effect of leupeptin on protein degradation in (a) normal human and (b) Swiss 3T3 fibroblasts. Cells were seeded (●, confluent; ▲, exponential growth), labelled, and degradation was measured as described in the Materials and methods section. Leupeptin was present only in the degradation medium. Points represent the means of four values ($\pm$ s.d. where greater than the span of the points).

period, indicating that at least some pepstatin had entered the cells even though it was insufficient to inhibit alone (results not shown).

Preincubation of normal human fibroblasts with pepstatin for 72 h before the measurement of degradation causes significant inhibition of proteolysis in confluent and in exponentially growing cultures (Fig. 2). Maximum inhibition occurred at a concentration of 100 $\mu\text{g/ml}$. An incomplete block of the increase in proteolysis normally occurring in cells approaching confluence was obtained. In contrast, preincubation of 3T3 cells with the inhibitor results in only slight inhibition of degradation in confluent cultures and no effect on exponentially growing cultures (Table 1). In some experiments, no inhibition was observed (Fig. 2), which may reflect different rates of uptake in different experiments. Although the inhibitory effects were slight, these studies with pepstatin are suggestive of lysosomal participation in proteolysis in both growth phases, and of the involvement of cathepsin D.

Huisman *et al.* (1973, 1974) have previously found that cysteinyl proteinases play a much larger part than cathepsin D in the digestion *in vitro* of native proteins by rat liver lysosomal enzymes at pH 5. Therefore we have studied the effect of the cysteinyl-proteinase inhibitor, leupeptin, on both Swiss 3T3 and normal human fibroblasts (Fig. 3). Leupeptin penetrates rapidly into cells, with maximum rates of inhibition being reached 4 h after presentation to fibroblasts, as found previously with mouse macrophages (Dean, 1979). Leupeptin has a marked inhibitory effect on degradation in both cell types, but does not completely block the increase in proteolysis normally occurring in cells approaching confluence. Inhibition was similar in both growth states for both cell types, indicating the participation of cysteinyl proteinases in degradation in both states. However, since leupeptin probably

permeates throughout the cell, its site of action is unclear.

Huisman *et al.* (1973, 1974) only obtained complete inhibition of degradation *in vitro* in the presence of inhibitors of both cysteinyl and aspartyl proteinases, e.g. iodoacetate and pepstatin. Therefore we have preincubated both Swiss 3T3 and normal human fibroblasts with pepstatin in the presence of leupeptin and were able to show a subsequent complete inhibition of the increase in proteolysis normally occurring in cells as they approach confluence (Table 1). Again, the 3T3 cells were less sensitive, and a higher concentration of leupeptin was needed to obtain this. The effect of both inhibitors presented simultaneously is greater than the sum of their effects when presented separately.

A further difficulty in interpreting work with leupeptin is that it can inhibit certain serine proteinases (Barrett, 1977). Therefore we have also conducted experiments with a more selective inhibitor of cysteinyl proteinases, Z-Phe-Ala-CHN₂, which has the additional advantage that its uptake into cells (like that of pepstatin) seems to be by pinocytosis, which directs it to lysosomes and excludes it from the cytosol. Thus it can be used to reveal specifically the action of lysosomal cysteinyl proteinases (Shaw & Dean, 1980). Fig. 4 shows that Z-Phe-Ala-CHN₂ can drastically inhibit proteolysis in both growth states in each cell type. Furthermore, it was able to block completely the increase in proteolysis at confluence even when present only in the degradation medium, suggesting that the lysosomal system may be largely responsible for the regulation. The much more rapid establishment of the effect of Z-Phe-Ala-CHN₂ than of that of pepstatin (above) may well be due to Z-Phe-Ala-CHN₂ entering by adsorptive endocytosis (Shaw & Dean, 1980), which permits faster uptake

Table 1. *Effect of pepstatin and leupeptin on protein degradation in (a) normal human and (b) Swiss 3T3 fibroblasts* Cells were incubated with the inhibitors for 72 h at the concentrations shown below. They were then trypsin treated and seeded into tissue-culture tubes, and labelling and degradation were performed in the presence of the inhibitors as described in the Materials and methods section. Results are means $\pm$ s.d. ($n = 4$).

	Concn. of inhibitors ($\mu\text{g/ml}$)		Degradation (%)	
	Pepstatin	Leupeptin	Exponential	Confluent
Fibroblasts (a) Human	0	0	30.48 $\pm$ 1.34	36.09 $\pm$ 0.61
	250	0	29.17 $\pm$ 0.65	33.90 $\pm$ 0.57
	0	250	23.32 $\pm$ 0.93	30.70 $\pm$ 0.93
	250	250	23.82 $\pm$ 1.14	24.13 $\pm$ 0.67
(b) Swiss 3T3	0	0	28.76 $\pm$ 0.71	31.19 $\pm$ 0.71
	250	0	28.09 $\pm$ 2.47	30.05 $\pm$ 0.60
	0	500	24.98 $\pm$ 0.81	28.26 $\pm$ 0.45
	250	500	23.21 $\pm$ 0.22	23.07 $\pm$ 0.30

Fig. 4. Effect of Z-Phe-Ala-CHN₂ on protein degradation in (a) normal human and (b) Swiss 3T3 fibroblasts. Cells were seeded (●, confluent; ▲, exponential growth), labelled, and degradation was measured as described in the Materials and methods section. Z-Phe-Ala-CHN₂ was present only in the degradation medium. Points represent the means of four values ($\pm$ S.D. where greater than the span of the points).

than that of fluid. Since Kirschke *et al.* (1980) have shown that cathepsin L is the most proteolytic of the presently characterized cysteinyl proteinases, it is noteworthy that Z-Phe-Ala-CHN₂ is a more effective inhibitor of cathepsin L than is leupeptin (Kirschke *et al.*, 1980), just as it is a more effective inhibitor of confluent proteolysis.

Taken together, the experiments above indicate that the lysosomal role in proteolysis is greater in confluent than in exponentially growing cells, under nutritionally complete conditions. This is consistent

with the observations of Hendil (1981) showing an increase in autophagy at high culture density of cells.

Although the sensitivities of 3T3 cells and human fibroblasts to inhibition by the permeating agents (NH₄Cl and leupeptin) were very similar, their sensitivities to the pinocytosed agents varied significantly. With both pinocytosed inhibitors (pepstatin and Z-Phe-Ala-CHN₂), 3T3 cells were considerably less sensitive than human fibroblasts, possibly because of differences in their rates of pinocytosis.

Thus we have defined three distinct conditions, namely incubation with NH₄Cl, exposure to Z-Phe-Ala-CHN₂, and preincubation with pepstatin plus leupeptin, that lead both to substantial inhibition of lysosomal proteolysis and also to the complete abolition of the difference in degradation rate between exponential-phase and confluent fibroblasts. Our evidence as a whole suggests that the regulation of degradation effected in confluent cultures is almost entirely a lysosomal function. As discussed in our previous work (Dean & Cockle, 1981; Cockle & Dean, 1982), this regulation may be a consequence of several factors besides the rate of cell division.

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