Distinct proteolytic mechanisms in serum-sufficient and serum-restricted fibroblasts

Transformed 3T3 cells fail to regulate proteolysis in relation to culture density only during serum-sufficiency

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1. Thymidine incorporation (reflecting cell division), degradation of long-half-life proteins and protein synthesis were compared in normal Swiss mouse 3T3 fibroblasts and their counterparts transformed by simian virus 40 at both high and low culture densities (no. of cells/cm²). 2. Normal cells maintained faster proteolysis at high culture density than at low. Degradation was in all conditions enhanced by serum deprivation (1% serum). In serum-sufficient (10%) conditions, there was an inverse correlation between degradation and cell division, but in serum-restricted conditions proteolysis increased substantially as culture density was increased, without change in cell division. Protein synthesis generally changed in a converse sense to protein degradation. 3. In serum-sufficient conditions, transformed 3T3 cells failed to regulate proteolysis in response to culture density. However, in serum-restricted conditions they can regulate proteolysis as do normal cells. Transformed 3T3 cells regulate protein synthesis and thymidine incorporation very poorly in response to culture density in both conditions studied. 4. The failure of regulation of both protein synthesis and degradation may contribute to the exaggerated growth of transformed cells in serum-sufficient conditions. The retention by such cells of regulation of proteolysis during serum restriction may also aid their survival. 5. Studies with several lysosomotropic agents indicated that lysosomes contribute to proteolysis in all conditions studied, but also that its regulation in serum restriction is distinct from that in serum sufficiency, and may involve primarily a non-lysosomal mechanism.

Many normal cell lines are known to regulate degradation of long-half-life proteins as they pass from the exponential growth phase into the stationary phase as confluence is reached: increases of up to 50% have been reported (Hendil, 1977; Tanaka & Ichihara, 1976; Dean & Cockle, 1981; Cockle & Dean, 1982a; Lockwood *et al.*, 1982). In contrast, transformed cells seem to have lost this capacity (Hendil, 1977; Cockle & Dean, 1982a; Lockwood & Minassian, 1982). Protein degradation is also regulated by hormones and nutrients (for review see Ballard, 1978) and can be increased markedly in cells in culture by removal of serum from the medium: such 'accelerated' proteolysis seems to be a function mainly of the lysosomal system (Dean, 1975; Amenta *et al.*, 1977).

Warburton & Poole (1977) have shown a close

Abbreviations used: Z-Phe-Ala-CHN₂, benzyloxy-carbonyl-phenylalanyl-alanyldiazomethane; SV40, simian virus 40.

inverse correlation between protein degradation and cell division by varying serum or growth-factor concentration in culture media. If changes in proteolysis with culture density (the number of cells/cm²) are similarly dependent on a change in the rate of cell division in the opposite sense, then this inability of transformed cells to control their proteolysis may be explained simply by their capacity for density-independent growth. Alternatively, as suggested by Warburton & Poole (1977) and Ballard *et al.* (1981), these two phenomena may be in a converse relationship, in which a change in the rate of proteolysis contributes to the determination of growth rate.

We have studied these possibilities in the present paper, by examining regulation of proteolysis with cell density in normal 3T3 and in their transformed counterparts. We have used both normal growth conditions and low-serum conditions, in which normal cells become quiescent at any cell densities owing to depletion of growth factors. We

have also used several lysosomotropic agents to examine lysosomal contributions to proteolysis in each condition (as described previously for normal cells in serum-sufficient conditions: Cockle & Dean, 1982b).

Materials and methods

These were as described previously (Cockle & Dean, 1982a,b), with the following additions. [6-³H]Thymidine (5Ci/mmol) was obtained from Amersham International (Amersham, Bucks., U.K.). Normal 3T3 and SV40-transformed 3T3 fibroblasts (Swiss mouse) were obtained from Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.).

General aspects of experimental design

In our previous study showing that transformed fibroblasts fail to regulate proteolysis in response to culture density (Cockle & Dean, 1982a), two different methods were used for studying such regulation. In the first, cells were grown either to confluence or to exponential phase, labelled, and their protein degradation was followed *in situ*. In the second, cells were prelabelled in standard conditions, resuspended by trypsin treatment and re-plated in tissue-culture tubes at the required range of densities for immediate study of proteolysis. This method had the advantage that any cells that detached from the monolayers but remained viable could be easily centrifuged into the cell layer when necessary during washing procedures, so that cells were not lost from the system during the progress of the experiments. Only the latter method was used in the present work, because with the transformed cells peeling of the cells from the flat tissue-culture surfaces was a persistent problem, which it was necessary to avoid. In addition, a continuous confluent layer of cells could be much more easily obtained in culture tubes than in dishes, which often leave areas in the centre virtually devoid of cells, especially after cells have been directly plated at very high culture density.

Degradation of long-half-life proteins was studied by the procedures that we described previously, with labelling for 16h before degradation experiments and for 24h for measurements of protein synthesis. In contrast, some other authors (e.g. Lockwood & Minassian, 1982; Lockwood *et al.*, 1982) used limit-labelling (selecting extremely long-half-life proteins) for proteolysis measurements, but short-term labelling (selecting for shorter-half-life molecules) for determination of protein synthesis.

Degradation was expressed as before as:

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

Cell lysis, measured as acid-precipitable material in the medium, was always less than 10%.

Protein and DNA synthesis

To measure protein synthesis, cells were treated in parallel and in an identical manner with those used for degradation measurements, with the exceptions that label was omitted from the tissue-culture flasks, and after a 3h period for adherence the medium was replaced with minimal essential medium containing 1% or 10% heat-inactivated foetal-calf serum, antibiotics and 0.1 μ Ci of [¹⁴C]-leucine/ml. Labelling was continued for the same time as degradation (24h). The cells were then washed five times with 10% trichloroacetic acid containing 10mM-leucine, and the precipitated cells were then dissolved in 90% formic acid. The whole of the resulting solution was counted for radioactivity, and protein synthesis was expressed as d.p.m. incorporated per 10⁴ cells.

For DNA-synthesis measurements, cells were treated in parallel and in an identical manner with those used for degradation, except that label was omitted from the tissue-culture flasks and, after the 3h period for cell adherence, the medium was replaced with minimal essential medium containing 1% or 10% heat-inactivated foetal-calf serum, antibiotics, 10mM-leucine, 5 μ g of thymidine/ml and 1 μ Ci of [³H]thymidine/ml. Decreasing the serum concentration to 1% was sufficient to restrict growth, and gave good cell viabilities; 0% serum was much less satisfactory in the latter respect. Labelling was continued for the same time period as degradation. The cells were then washed five times with 10% trichloroacetic acid containing 5 μ g of thymidine/ml, and the precipitated cells were then dissolved in 90% formic acid. The whole cell fraction was taken for scintillation counting, and DNA synthesis was also expressed as d.p.m. incorporated/10⁴ cells.

For both protein and DNA synthesis it was necessary to centrifuge the cell fractions at 3000g for 10min between washes, and for this reason 0.1ml of 10% (w/v) bovine serum albumin was added before the trichloroacetic acid to these tubes.

Background incorporation of both leucine and thymidine radioactivity was measured in a variety of different conditions, and in all cases was between 75 and 200 d.p.m./10⁴ cells. Results presented are means $\pm$ s.d. for individual experiments done in quadruplicate and are representative of several different experiments.

Fig. 1. Protein degradation (a), protein synthesis (b) and DNA synthesis (c) at various cell densities in normal (i) and SV40-transformed (ii) 3T3 fibroblasts in 1% (▲) and 10% (●) serum

Cells were treated as described in the Materials and methods section, the same batch of cells being used for measurement of the three parameters. The same labelling medium was used for both types of cells, so that all results are directly comparable. Points represent means $\pm$ S.D. ($n = 4$).

Results and discussion

Protein turnover and growth of normal 3T3 fibroblasts

As described previously (Cockle & Dean, 1982a), protein degradation increases gradually but significantly with culture density in normal 3T3 fibroblasts in 10% serum (Fig. 1ai). Between cell densities of 5×10^4 and 20×10^4 /tube there is a

very close inverse correlation between protein degradation and DNA synthesis (Figs. 1ai and 1ci), in agreement with the results of Warburton & Poole (1977).

On replacement of normal medium with medium containing only 1% serum, cells remain viable throughout the 24h experiment, as judged by insoluble radioactivity released into the medium, which remains less than 10%. Low-serum

conditions depress DNA synthesis considerably (Fig. 1ci), and incorporation of radioactivity remains constant and close to background values over the cell-density range 5×10^4 – 20×10^4 cells/culture. A slightly raised incorporation is observed at the lowest density of 2.5×10^4 cells/culture, which may indicate that there are sufficient growth factors in 1% serum to stimulate growth at this density. In spite of constant DNA synthesis in the density range 5×10^4 – 20×10^4 cells/culture, protein degradation increases gradually but significantly over the same range in 1% serum. Degradation values at all cell densities are higher in 1% than in 10% serum, which is an example of 'accelerated' proteolysis owing to hormonal depletion. It is interesting that, although the proteolysis curve with 1% serum is deflected upwards in relation to that for 10% serum, the curves are almost parallel over the range of cell densities used.

Thus, surprisingly, in 1% serum the regulation of proteolysis with cell density can occur independently of changes in cell division; it is therefore unlikely that changes in proteolysis are secondary to those of cell division.

Protein synthesis (Fig. 1bi) changes with respect to cell density in the opposite sense to protein degradation. Such a change is expected, because protein accumulation as cells become quiescent at confluence must approach zero (unless there is accelerated cell detachment and death). Since protein-synthesis rates are large in exponential growth to provide a net protein gain, they must be drastically decreased concurrently with an increase in proteolysis. Decreases in protein synthesis (65% in 10% serum; 70% in 1% serum) are greater than increases in protein degradation (40% in 10% serum; 31% in 1% serum) over the range of cell densities 2.5×10^4 – 20×10^4 cells/culture. The protein-synthesis curve in 1% serum is deflected down with respect to that in 10%, but, like the degradation curve, remains approximately parallel over the range of cell densities used here. No significant changes in cell viability or detachment occur.

Protein turnover and growth of SV40-transformed 3T3 fibroblasts

No significant change in protein degradation in the range 2.5×10^4 – 15×10^4 cells per tube was observed for the transformed cells growing in 10% serum (Fig. 1aii), as described previously for other transformed cells (Cockle & Dean, 1982a). There was a small increase in degradation at a cell density of 20×10^4 /tube, but this regulation was very slight compared with that observed in normal fibroblasts over the same range (Fig. 1ai). In addition, rates of degradation in transformed cells at all densities in 10% serum were significantly lower than rates of

degradation in normal cells even at the lowest cell densities, 2×10^4 . These results are consistent with those of Gunn *et al.* (1977) and Ballard *et al.* (1980), although low degradation rates may not be universal in transformed cells (Baxter & Stanners, 1978; Cockle & Dean, 1982a). It is possible that lower rates of degradation are achieved in normal cells at densities below 2.5×10^4 cells/tube.

Unexpectedly, transformed cells grown in 1% serum show a gradual but significant regulation of degradation over the range of cell densities used (Fig. 1aii). The response is very similar to that for normal cells in 10% serum, comprising an increase in degradation from lowest to highest cell density of 44% (cf. the corresponding value, 40%, for normal cells in 10% serum). Accelerated proteolysis (owing to removal of most serum) occurs in the transformed cells, and its magnitude varies with cell density (2.5×10^4 cells, 14%; 20×10^4 cells, 50%). Previous suggestions that proteolysis in transformed cells is less susceptible to serum depletion (Ballard *et al.*, 1981) are thus an oversimplification, and only hold at low cell densities for SV40-transformed 3T3 cells.

In contrast with normal cells, transformed cells in 10% serum show no significant decrease in DNA synthesis over the entire range of cell densities (Fig. 1cii), revealing their ability for density-independent growth; in addition, thymidine incorporation is similar to that in normal cells at the lowest cell density. DNA synthesis is less in 1% than in 10% serum, and shows a small but significant decrease at higher densities (15×10^4 – 20×10^4 cells/tube). Nevertheless, even at the highest density DNA synthesis is considerably higher than in normal cells. These findings exemplify the idea that growth of transformed cells is less sensitive to changes in growth-factor concentration than is that of normal cells.

It is interesting that a response of proteolysis to changing cell density is observed for both transformed and normal cells in 1% serum. This implies that this proteolytic change is not consequent on changes in cell growth.

Protein synthesis in the transformed cells growing in 10% serum changes only slightly over the density range studied, but there is a larger decrease (approx. 12%) in the presence of 1% serum (Fig. 1bii). This decrease is considerably smaller than that of normal cells in either serum condition.

The results together suggest that the continued growth and therefore net protein accumulation with increasing cell density of transformed cells growing in serum-sufficient conditions is accompanied by a lower capacity for regulation of both protein degradation and synthesis. In 1% serum, regulation of proteolysis in these cells is almost as effective as that of normal cells, whereas the de-

crease in protein synthesis with increasing density is much smaller than that in normal cells; thus changes in DNA synthesis are much slighter than those of normal cells. That proteolysis can be regulated without changes in cell division re-emphasizes the need to consider further whether it can contribute to, rather than only respond to, changes in cell growth. In the former case, local pharmacological control of protein degradation could have implications in the management of some cancers.

Lysosomal contributions to degradation

One possible explanation of the surprising findings above is that two different regulatory mechanisms operate on protein degradation in serum-restricted and -sufficient conditions, but the transformed cells only retain that which can operate in restricted conditions. We have previously presented evidence implying that lysosomes play a major role in the regulation of proteolysis with respect to cell density during serum-sufficiency (Cockle & Dean, 1982b). In order to investigate this in transformed cells, we have studied the effect of the lysosomotropic agent NH_4Cl (Fig. 2) and the group-specific proteinase inhibitors Z-Phe-Ala-

CHN_2 (Fig. 3) and leupeptin (Fig. 4) on degradation in cultures at low and high density in both serum concentrations.

NH_4Cl seems to inhibit intracellular proteolysis by raising the intralysosomal pH (see Seglen *et al.*, 1981). As found previously (Cockle & Dean, 1982b), it has a marked inhibitory effect on protein degradation in normal 3T3 cells in 10% serum: inhibition is greater in confluent than in subconfluent cultures, such that the regulation in degradation is completely abolished at 20 mM amine (Fig. 2bi). In contrast, in normal cells in 1% serum (Fig. 2ai), there is only a partial decrease in the regulation in cells at high density, although inhibition is marked at both cell densities.

NH_4Cl also has a marked and similar inhibitory effect on proteolysis in the transformed 3T3 cells in 10% serum (Fig. 2bii). The slight increase in degradation observed in the confluent cells in the absence of the amine is completely inhibited by 20 mM- NH_4Cl . In contrast, in the transformed cultures in the serum-depleted state, this amine cannot equalize rates of degradation in the two culture densities, although it is inhibitory at each.

These results indicate again that the lysosomal

Fig. 2. Effect of NH_4Cl on protein degradation in normal (i) and SV40-transformed 3T3 (ii) fibroblasts at low (L; ▲) and high (H; ●) cell densities in the presence of 1% (a) or 10% (b) serum

Cells were labelled, washed and seeded at 10^4 (L) and 10^5 (H), and 2×10^4 (L) and 2×10^5 /tube (H), for normal and transformed cells respectively. Degradation was measured over 24 h as described in the Materials and methods section, and results are means $\pm$ s.d. ($n = 4$). The degradation values for the normal cells in 1% serum at the two densities are significantly different in 30 mM- NH_4Cl at the 1% level (as judged by a two-tailed Student's *t* test), and in 50 mM- NH_4Cl the difference is significant at the 5% level. For the transformed cells in 1% serum, the difference remains significant at the 1% level even for cultures in 50 mM- NH_4Cl .

Fig. 3. Effect of Z-Phe-Ala-CHN₂ on protein degradation in normal (i) and transformed (ii) 3T3 fibroblasts in low (L; ▲) and high (H; ●) culture densities in the presence of 1% (a) and 10% (b) serum

Cells were labelled, washed and seeded at 10^4 (L) and 10^5 (H) per tube, and 2×10^4 (L) and 2×10^5 (H) per tube, for normal and transformed cells respectively. Degradation was performed for 24h as described in the Materials and methods section, and results are means $\pm$ s.d. ($n = 4$).

system may be responsible for the regulation in degradation at confluence in serum-sufficient normal cultures. However, the inability of NH₄Cl to abolish the regulation in 1% serum suggest that a non-lysosomal component is involved. The similar response of normal and transformed cells in 1% serum to NH₄Cl suggests that similar degradative mechanisms may be in action in each under these conditions. The derangement of degradation in transformed 3T3 cells (Cockle & Dean, 1982a) is expressed primarily in 10% serum, where regulation essentially fails, again implying that mechanisms in low and high serum concentrations are distinct.

The lysosomotropic cysteine-proteinase inhibitor Z-Phe-Ala-CHN₂ (Shaw & Dean, 1980) causes a substantial inhibition of degradation at both cell densities in normal 3T3 cells in 10% serum, and equalizes degradation rates in the two culture densities when given at 100 μ g/ml (Fig. 3bi; Cockle & Dean, 1982b). It also greatly decreases degradation at each cell density in normal cells in 1% serum, though rates at the two densities are not equalized (Fig. 3ai). The inhibitor decreases degradation in SV40-transformed 3T3 cells essentially as it does in normal cells under all conditions studied, and again fails to equalize degradation rates in the cultures in 1% serum. The results with

Fig. 4. Effect of leupeptin on protein degradation in normal (i) and transformed (ii) 3T3 fibroblasts in low (L; ▲) and high (H; ●) culture densities in the presence of 1% (a) and 10% (b) serum. Cells were labelled, washed and seeded at 10⁴ (L) or 10⁵ (H) per tube, or 2 × 10⁴ (L) and 2 × 10⁵ (H), for normal and transformed cells respectively. Degradation was measured over 24 h, and the points represent means ± S.D. (n = 4).

Table 1. Characteristics of protein degradation in normal and transformed 3T3 fibroblasts

Cell line	[Serum] (v/v)	Is proteolysis increased when cultures become confluent?	Can lysosomotropic agents completely prevent the increase at confluence?		
			NH ₄ Cl	Z-Phe-Ala-CHN ₂	Leupeptin
Normal	1%	Yes	No	No	No
	10%	Yes	Yes	Yes	No
Transformed	1%	Yes	No	No	No
	10%	No	—	—	—

this inhibitor substantiate those with NH₄Cl in indicating that the mechanisms of degradation in serum-restricted conditions may be distinct from those in serum sufficiency in normal cells, and in implicating a non-lysosomal component in regulation in 1% serum in both normal and transformed cells.

As previously (Cockle & Dean, 1982b), leupeptin inhibited breakdown in normal cells less substantially than did either of the other agents; the same was true for SV40-transformed 3T3 cells (Fig. 4). In addition, whereas NH₄Cl and Z-Phe-Ala-CHN₂ were more effective in 10%- than in 1%-serum containing cultures, so that only in the

former could they equalize breakdown rates in subconfluent and confluent cells, leupeptin showed no such differential effect. It could not overcome the density-dependent breakdown regulation in 1% serum by either cell type.

Table 1 gives a qualitative summary of the characteristics of degradation in the conditions under study, and the effects of the three inhibitors studied. This summary makes more readily apparent the indications that density-dependent regulatory mechanisms in serum-sufficient and serum-restricted conditions differ, that transformed 3T3 cells have lost the former, but not the latter, regulatory mechanism, and that lysosomes are of prime concern in the former regulation, whereas some non-lysosomal mechanism may be more important in the latter.

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References

- Amenta, J. S., Sargus, M. J. & Baccino, F. M. (1977) *Biochem. J.* **168**, 223–227
- Ballard, F. J. (1978) *Essays Biochem.* **13**, 1–37
- Ballard, F. J., Wong, S. S. C., Knowles, S. E., Partridge, N. C., Martin, T. J., Wood, C. M. & Gunn, J. M. (1980) *J. Cell Physiol.* **105**, 335–346
- Ballard, F. J., Nield, M. K., Francis, G. L. & Knowles, S. E. (1981) *Acta Biol. Med. Germ.* **40**, 1293–1300
- Baxter, A. C. & Stanners, C. P. (1978) *J. Cell. Physiol.* **96**, 139–146
- Cockle, S. M. & Dean, R. T. (1982a) *Biosci. Rep.* **2**, 107–114
- Cockle, S. M. & Dean, R. T. (1982b) *Biochem. J.* **208**, 795–800
- Dean, R. T. (1975) *Nature (London)* **257**, 414–416
- Dean, R. T. & Cockle, S. M. (1981) *Acta Biol. Med. Germ.* **40**, 1571–1575
- Gunn, J. M., Clark, M. G., Knowles, S. E., Hopgood, M. F. & Ballard, F. J. (1977) *Nature (London)* **266**, 58–60
- Hendil, K. B. (1977) *J. Cell. Physiol.* **92**, 353–364
- Lockwood, T. D. & Minassian, I. A. (1982) *Biochem. J.* **206**, 251–258
- Lockwood, T. D., Minassian, I. A. & Roux, L. (1982) *Biochem. J.* **206**, 239–249
- Seglen, P. O., Gordon, P. B., Grinde, B., Solheim, A., Kovacs, A. L. & Poli, A. (1981) *Acta Biol. Med. Germ.* **40**, 1587–1598
- Shaw, E. & Dean, R. T. (1980) *Biochem. J.* **186**, 385–390
- Tanaka, K. & Ichihara, A. (1976) *Exp. Cell Res.* **99**, 1–6
- Warburton, M. J. & Poole, B. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2427–2431