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LYSOSOMAL ENZYMES AND AGING *IN VITRO*: SUBCELLULAR ENZYME DISTRIBUTION AND EFFECT OF HYDROCORTISONE ON CELL LIFE-SPAN*

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SUMMARY

The acid phosphatase and β glucuronidase activities of four subcellular fractions (nuclear, mitochondrial-lysosomal, microsomal, supernatant) of WI–38 cells were compared during *in vitro* aging. All of the fractions showed an age-associated increase in activity. The increase in the lysosomal fraction was sufficient to account for the increase in the whole homogenate. The supernatant fraction showed a consistent and pronounced increase suggesting a decrease in latency.

Hydrocortisone stabilized the lysosomes to some extent. However the presence of hydrocortisone (5 μg/ml) in the growth medium consistently extended the life-span of the culture 20–30%. The magnitude of the extension seemed to be directly proportional to the amount of time the cultures were exposed to the added hormone.

INTRODUCTION

It is now generally well established that human diploid cell lines in culture have a finite life-span. During serial subcultivation of these lines, their capacity to proliferate declines progressively until finally the culture can no longer be subcultivated and is lost[1,2]. This system has received much attention as a model for the study of aging at the cellular level and has been used in a variety of studies both of the regulation of cell proliferation and of the changes that accompany declining proliferative capacity[3].

In previous studies with this system, we reported a constant increase with age in the specific activities of acid phosphatase (EC 3.1.3.2) and β glucuronidase (EC 3.2.1.31), two enzymes usually associated with lysosomes[4–6]. Confirmation of this finding has been forthcoming both at the biochemical level[7,8] and in ultrastructural studies both from our laboratory and others[9–12].

Lysosomal enzymes exhibit structurally-linked latency and possible explanations for the overall increase in the specific activity of these enzymes during aging in-

* Preliminary reports of portions of this paper have been presented elsewhere[5,6].

clude: (1) A real increase in lysosomal enzyme concentration in the cells due to increased synthesis, decreased degradation, and/or various combinations of both. Recently, Milisauskas and Rose[8] showed increased amounts of immunoprecipitable lysosomal enzymes in older populations; (2) Various activation phenomena as, for example, an increase in the fragility of the lysosomal membrane, that would, under our conditions of homogenization, permit more rapid activation of the enzyme with a resulting increased reaction rate; (3) The possibility suggested by our studies on the heterogeneity of the molecular form and localization of acid phosphatase that the increase in acid phosphatase may not be exclusively lysosomal, but may occur in other subcellular fractions[13]; (4) Various combinations of the above.

To investigate these possibilities, we have carried out a series of experiments designed to determine the specific activity of acid phosphatase and β glucuronidase in various subcellular fractions of WI–38 cells during aging. We have also attempted stabilization of these lysosomal enzymes in older cultures with hydrocortisone and have studied the effect of hydrocortisone on the aging process *in vitro*. The results of these studies comprise the basis for this report.

MATERIALS AND METHODS

Starter cultures of human diploid cell strain WI–38[2] were obtained from Dr. L. Hayflick of Stanford University. The methods of subcultivation and harvesting and preparation of the cells for enzyme assays were as described elsewhere[4,13,14].

For preparation of the various subcellular fractions, all operations were carried out at, or near, 4°C. The cells were suspended in 10^{-3} M phosphate buffer at pH 7.2 and stirred gently for 10 min. This treatment was followed by homogenization with a Dounce apparatus using, first, a loose and then a tight-fitting pestle. Microscopic examination of random samples of the homogenate showed that this treatment resulted in virtually complete cell breakage. Immediately following the homogenization, 0.5 M sucrose was added to the mixture to give a final sucrose concentration of 0.25 M. The various fractions were then separated by centrifugation as described below and four subcellular fractions were prepared: (1) a nuclear fraction that was sedimented at 500 $\times$ *g* for 10 min; (2) a lysosomal-mitochondrial fraction sedimenting at 12 500 $\times$ *g* (20 min); (3) a microsomal fraction that was sedimented at 105 000 $\times$ *g* (30 min); and (4) the supernatant. Following separation, each fraction was treated with Triton X-100 (1% final concentration), and the enzyme activity was then assayed.

As we have reported previously[13], this method of preparation consistently allows greater than 95% of the activity in the whole homogenate to be recovered in the subcellular fractions. Approximately 50% of the specific activity is recovered in the lysosomal fraction (40–50% of total activity) while only about 15% is recovered in the supernatant (105 000 $\times$ *g*) fraction.

In other experiments, also previously reported[13], we have shown that treatment of the lysosomal fraction with Triton X-100 (1%) in 0.25 M sucrose with stirring and subsequent centrifugation at 105 000 $\times$ *g* resulted in 93% of the lysosomal activity appearing in the supernatant. Similar treatment with 0.25 M sucrose alone consistent-

ly resulted in less than 25% of the activity released into the supernatant. Thus, this method allows isolation from human diploid cells of a subcellular fraction which meets the biochemical criteria for lysosomes; this fraction contains the greatest proportion of acid phoshatase activity; in terms of specific activity this fraction is enriched over the crude homogenates; the activity is sedimentable and membrane bound; and once released by a detergent the activity is no longer sedimentable.

Acid phosphatase was determined by an adaptation of the method of Bessey, Lowry and Brock[15] which depends on the enzymatic hydrolysis of *p*-nitrophenylphosphate. Aliquots of the appropriate homogenate fractions were incubated for 30 min at 37°C in 0.05 M citrate buffer (pH 5.0) which contained 6.1 mM substrate. The reaction was stopped by the addition of alkali and the *p*-nitrophenol measured spectrophotometrically at 410 nm (mμ). β Glucuronidase was measured at pH 4.5 according to the method of Fishman[16] using phenolphthalein glucuronide as substrate.

For studies on lysosomal stabilization with hydrocortisone, the lysosomal fractions were prepared as described above except without Triton X-100 treatment. At various time intervals, in a series of replicates, the particulate fraction was removed by centrifugation and the supernatant assayed for activity as above.

In determining the effects of hydrocortisone on life-span, parallel cultures were started with and without hydrocortisone, and at appropriate intervals cultures were harvested, the cells counted with a hemocytometer or Coulter counter and cumulative population doublings were then calculated.

RESULTS

Table I summarizes our studies on the subcellular distribution of acid phosphatase activity in populations at three passage levels. All of the fractions showed an increase in activity with aging; the nearly 40% increase in the lysosomal fraction was sufficient to account for the increase in the whole homogenates noted previously[4]. The supernatant fraction, however, also showed a pronounced increase in activity and the

TABLE I

THE SUBCELLULAR DISTRIBUTION OF ACID PHOSPHATASE ACTIVITY IN WI-38 CELLS DURING AGING

Passage level	*Specific activity (mμmoles/min per mg protein)**			
	Nuclear fraction	*Lysosomal fraction*	*Microsomal fraction*	*Supernatant fraction*
17–25	21.33 ± 1.49 (4)	64.07 ± 6.46 (4)	22.20 ± 6.32 (4)	14.68 ± 2.15 (4)†
26–35	24.48 ± 2.24 (4)	69.58 ± 6.46 (4)	35.70 ± 6.23 (4)	17.31 ± 3.98 (4)
36–50	27.28 ± 2.91 (9)	89.59 ± 11.2 (9)	41.86 ± 6.90 (9)	21.90 ± 0.72 (9)†

* The values show the mean ± standard error of the mean followed by the number of determinations in parentheses.

† Difference significant at $p < 0.005$.

TABLE II

THE SUBCELLULAR DISTRIBUTION OF β GLUCURONIDASE ACTIVITY* IN WI-38 CELLS DURING AGING

Passage level	*Nuclear fraction*	*Lysosomal fraction*	*Microsomal fraction*	*Supernatant fraction*
17–25	11.86 ± 1.27 (4)	42.28 ± 8.58 (4)	12.42 ± 3.12 (4)	5.99 ± 1.08 (4)†
26–35	13.92 ± 2.73 (4)	58.84 ± 3.62 (4)	17.71 ± 3.91 (4)	11.67 ± 1.65 (4)
36–50	18.32 ± 1.89 (9)	70.24 ± 9.45 (9)	25.15 ± 3.22 (9)	14.04 ± 1.60 (9)†

* In terms of specific activity, μg/hour per mg protein. The values show the mean ± the standard error of the mean followed by the number of determinations in parentheses.

† Difference significant at $p < 0.01$.

difference between the earliest and latest passage groups in this fraction was significant at $p < 0.005$.

Similar analysis of the β glucuronidase activity (Table II) gave similar results. The lysosomal activity increased nearly 70% while the supernatant fraction showed a better than 2-fold difference between the earliest and the latest passage groups ($p < 0.01$). Since protein distribution per fraction did not shift significantly during aging, the data indicate a disproportionate quantity of these two enzymes in the nonparticulate fraction of senescent cells and suggest the possibility that an increase in the mobility (decrease in latency) of acid phosphatase and β glucuronidase may account in part for the increased activity of this fraction. As stated previously, however, this increase could also be due to increased activity of the non-lysosomal enzyme activity in this fraction.

One method for stabilizing lysosomal membranes is with hydrocortisone[17,18]. To determine whether stabilization was effective in these cell cultures, two kinds of experiments were done. In the first, homogenates were prepared from cells at different passages as described under Methods, and replicate tubes of homogenate with and without hydrocortisone (5 μg/ml) were incubated at room temperature for various periods of time. The sample tubes were then centrifuged and the supernatant assayed for acid phosphatase and β glucuronidase. The results for acid phosphatase are shown in Fig. 1 (the results for β glucuronidase were parallel and are not shown). For all passages ranging from 23 to 51, hydrocortisone reduced the rate of leakage of enzyme into the nonparticulate fraction. The magnitude of the hydrocortisone effect appeared to be highest for the younger populations (passages 23 and 30) and the amount of enzyme released into the supernatant lowest in the older populations (passages 35 and 51). However, this difference between young and old populations may simply be a reflection of the lower amount of particulate-bound enzyme present immediately after homogenization in the older populations. In fact, for these experiments, in the younger passage level cells, approximately 70% of the activity was in the pellet at zero time while in the oldest group only 58% was recovered. For all groups, however, the total enzyme activity (pellet + supernatant) never fell below 81% of the zero time activity.

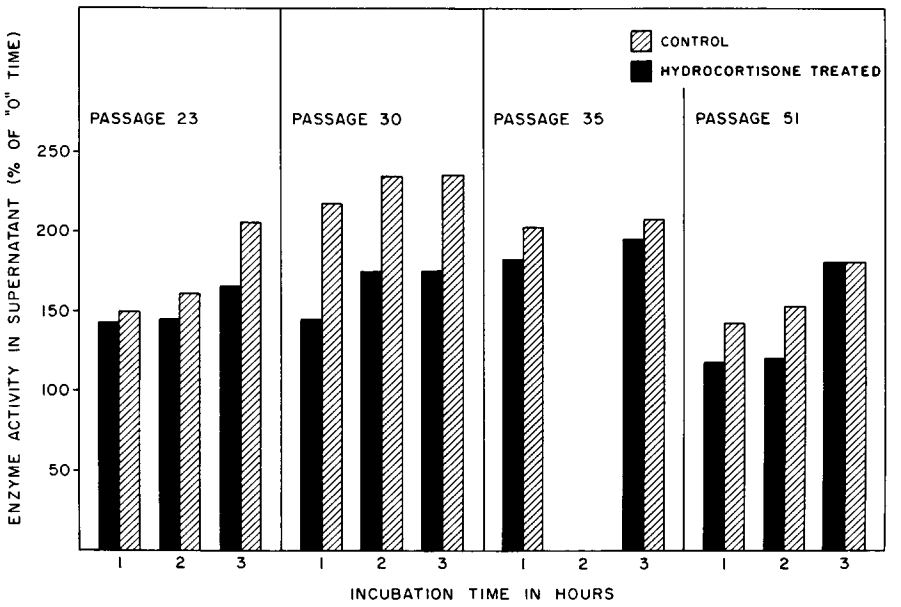

Fig. 1. Acid phosphatase activity in supernatant from lysosome preparation after various time periods. Activity in supernatant at zero incubation time is taken as 100.

In the second type of experiment, designed to evaluate the effect of lysosome stabilization, cultures were prepared and grown in duplicate with and without hydrocortisone (5 μg/ml). After different periods in culture, total acid phosphatase and β glucuronidase activities were determined, as described under Methods, in the crude homogenate and in the various subcellular fractions. No hydrocortisone was present in the incubation medium prepared for the assays. The results confirmed our earlier finding[4] that the activity of these enzymes, as assayed in crude homogenates, increased with aging. However, whether hydrocortisone was present or not, there was still a gradual increase in the enzyme activity. In addition, the subcellular distribution of acid phosphatase and β glucuronidase was not affected by the hydrocortisone treatment (Table III). The indices for the distribution of acid phosphatase were alike in hydrocortisone-treated and control cultures (similar results were obtained for β glucuronidase and therefore the data are not shown). Cultures grown in the presence of hydrocortisone, however, showed a striking prolongation of their life-span. In this series, hydrocortisone-treated cultures lived 60 passages while controls lived only 45 passages (Table III). In other experiments in which cultures were carried in duplicate, with and without the addition of hydrocortisone (5 μg/ml), from passage levels in the very early 20's to the end of the life-span, the highest passages attained in the presence and absence of hydrocortisone, respectively, were 66 *vs.* 54, 52 *vs.* 43, and 54 *vs.* 45.

This extension of the life-span could be shown on the basis of actual cell numbers, as well as the number of subcultivations. Figure 2 summarizes the results of one of our experiments in which cell yield/vessel is plotted as a function of passage. For this study, cells were subcultured at a ratio of 1:4 (2 doublings = 2 passages) each

TABLE III

THE EFFECT OF HYDROCORTISONE ON THE SUBCELLULAR DISTRIBUTION OF ACID PHOSPHATASE ACTIVITY* IN WI-38 CELLS DURING AGING

Passage number	*Subcellular fraction*							
	Nuclear		*Lysosomal*		*Microsomal*		*Supernatant*	
	H[a]	*C*[b]	*H*	*C*	*H*	*C*	*H*	*C*
34	19.0	13.7	59.8	53.6	9.0	21.7	12.2	11.0
37	23.2	20.9	45.6	44.4	17.8	20.2	13.6	12.5
42	20.8	24.4	43.1	51.9	21.8	9.7	14.3	14.1
45	19.5	15.5	45.8	39.9	20.0	28.7	14.7	15.9
Mean 34–45	20.6	18.6	48.6	47.5	17.2	20.1	13.7	13.4
48	16.6	—[c]	48.3	—	20.7	—	14.4	—
54	24.1	—	54.1	—	14.9	—	6.9	—
58	9.9	—	49.2	—	31.2	—	9.7	—
60	16.8	—	49.3	—	22.4	—	11.6	—
Mean 34–60	18.7	—	49.4	—	19.7	—	12.2	—

* In terms of percent specific activity in the fraction; *i.e.*, for any fraction, the percent specific activity is derived from the expression:

$$\frac{\text{m}\mu\text{moles/min per mg protein (fraction)}}{\text{m}\mu\text{moles/min per mg protein (all fractions)}} \times 100$$

[a] H = Hydrocortisone (5 μg/ml) treated.
[b] C = Control — no added hydrocortisone.
[c] Phased out.

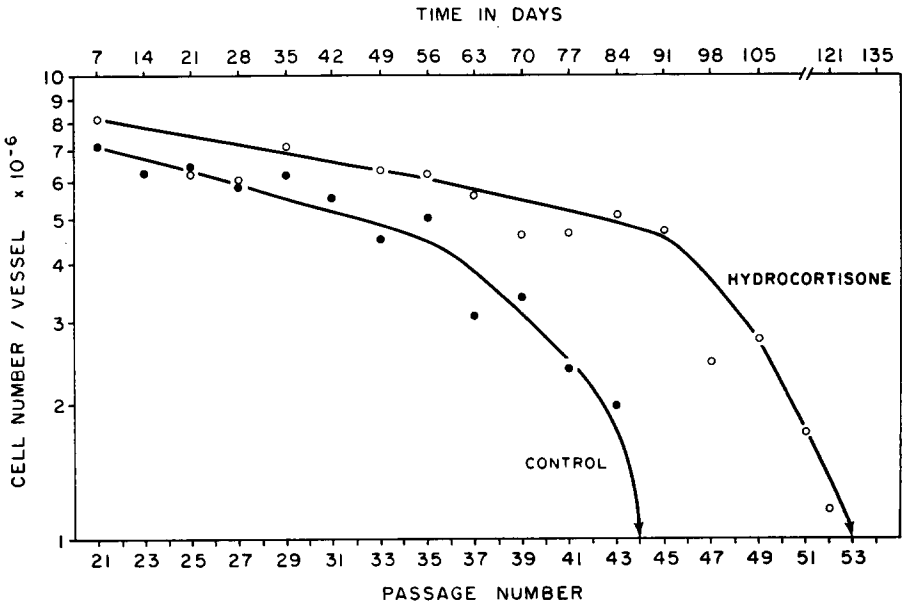

Fig. 2. Cell yield per vessel as a function of serial passage number or time in days for hydrocortisone (cortisol) treated and control cultures. The points where the arrows intersect the abcissa represent the passage at phase out.

week. As the cells approached the end of their life-span, the amount of time necessary to achieve confluency became extended, and the cells were fed with fresh medium each week, but subcultivated only when they had reached confluency. When more than one week was required to attain confluency, the next subcultivation was made at a 1:2 ratio (1 doubling = 1 passage). The points in Fig. 2 where the curves intersect the abscissa (arrows) indicate the passage level at which the cells, after repeated refeedings with fresh medium were nevertheless unable to proliferate and achieve confluency. The magnitude of the extension of the life-span is striking since the treated cultures were still proliferating 6 weeks after the controls had phased out.

The effect of adding, or adding and then removing, hydrocortisone at different passage levels was tested (Table IV). As can be seen, the earlier in the life cycle of the culture that the hormone was added, the greater the increase in lifespan. When the hormone was added at passage 21 and removed at later passages, a similar positive relationship was found between the presence of hydrocortisone and the increased life-span of the culture.

TABLE IV

THE EFFECT OF ADDITION AND REMOVAL OF HYDROCORTISONE AT DIFFERENT PASSAGES ON LIFE-SPAN

Passage exogenous hormone present	*Passages achieved at phase out*	*% Increase in life-span in presence of hormone*
None	45	None
44–45	45	None
39–49	49	9
29–50	50	11
21–54	54	20
21–45	52	16
21–39	50	11
21–29	48	6

DISCUSSION

Perhaps the best documented of the age-associated functional changes in diploid cells is the increase in lysosomes and lysosomal enzyme activity[4,9–12]. These organelles have been implicated in a wide variety of degenerative processes including aging[19,20]. The increase in lysosomal activity may result from age-associated changes in the lysosomal membrane structure permitting leakage of lysosomal hydrolases into the cell. Alternatively, the orderly breakdown of lysosomal structures and release of their contents may be required for the normal cellular functioning and an inability to break down in old cells would lead to an accumulation of lysosomes. As we have suggested previously[3], either of these could result in the inability of the cells to initiate DNA synthesis and/or cell division and a condition of unbalanced growth could result leading eventually to cell death.

The initial purpose of these experiments was to isolate lysosomal fractions and

to determine some of the characteristics of these fractions in young and old cells. Although the preparations described are relatively crude, we had shown previously that they satisfy many of the biochemical criteria for lysosomes such as enzyme spectrum, enzyme latency, and inhibition sensitivity[13].

Previous work of ours had shown an increase in enzyme activity[4]. More recently Milisauskas and Rose[8] showed an increase in the cellular content of several lysosomal enzymes as determined immunologically. Although the results reported here agree with these previous studies, the large increase in activity in the nonparticulate fraction suggests that a decrease in enzyme latency may also be occurring during aging. This finding would support the hypothesis that leakage of lysosomal enzymes during aging causes damage to cellular organelles. An alternative explanation is there is an age-associated increase in the cellular content of nonlysosomal acid phosphatase. In the latter case, the increased nonparticulate enzyme activity could be independent of lysosomal function. We are attempting to resolve these alternatives.

Our results with hydrocortisone showing stabilization of isolated lysosomal fractions are in agreement with the results of Weissmann and Dingle[17], DeDuve[18] and others. However, the absence of changes in the subcellular distribution of lysosomal enzymes in preparations from cells grown in the presence of the hormone suggest the possibility that the effectiveness of the hormone in extending life-span may be unrelated to its effect on lysosomes.

Hydrocortisone, cortisone and other corticosteroids have been reported to prolong the *in vitro* survival time of several cell types[21–23]. These studies have been concerned with postmitotic maintenance of the cultures. Macieira-Coelho[24], however, was the first to report the increase in proliferative life-span of diploid fibroblast-like cells in culture. Our data are in agreement with his and show a true increase in the number of population doublings that occur in corticosteroid-treated cultures. Data published elsewhere[25,26] indicate that this increase is not due to an increase in the number of cells sticking to the surface or improved maintenance during post-confluency. Rather, they show that in the presence of the steroid, an actual increase in cell division (in the population) results in increased cell yields. We have also shown that the effect is due, in part, to an increased number of cells in the proliferating pool[27,28].

Growth inhibition of tissue culture cells by hydrocortisone has been reported by a number of authors[2]. However, effects with hydrocortisone similar to ours have been reported by Prince and Castor[30] for cartilage cells and by Smith *et al.*[31] for human fetal lung cells.

Recently, Thrash and Cunningham[32] have shown the stimulation of division by hydrocortisone in density-inhibited 3T3 cells and Armelin[33] and Gospodarowicz[34] have shown that hydrocortisone amplifies the activity of the pituitary and brain-derived polypeptides which stimulate cell division.

The mechanism by which this growth enhancement occurs remains obscure. One can speculate that it may be related to the interaction of the hormone with the lysosomes. Alternatively, the hormone may act on chromatin and modify transcription. Finally, the effect may occur at the cell surface. Experiments are now underway in our laboratory to resolve these various possibilities.

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