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EFFECTS OF HYDROCORTISONE AND ASPIRIN ON PROTEIN SYNTHESIS AND POST-TRANSLATIONAL PROTEIN MODIFICATION IN CULTURED CELLS*

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Abstract—Arginyl-tRNA transferase is suggested to function as a post-translational modifier of proteins through the addition of arginine to the NH_2-terminus of specific acceptor proteins. Both hydrocortisone and aspirin produced an age-dependent stimulation of protein synthesis in normal human fibroblasts (IMR90), while producing an inhibition of protein synthesis in SV40 virus-transformed IMR90 cells. The effect of aspirin was confined primarily to the cytoplasmic compartment, whereas hydrocortisone produced its effect at both cytoplasmic and nuclear levels. Neither hydrocortisone nor aspirin had a direct effect on arginyl-tRNA transferase activity *in vitro*; however, hydrocortisone resulted in a marked increase in the availability of chromosomal proteins subject to modification by arginyl-tRNA transferase. This stimulatory effect was attenuated by increasing culture age. The modified chromosomal proteins were found to be dissociated from native chromatin, suggesting that arginylation either triggered their release or prevented reassociation with chromatin thereafter. Hydrocortisone produced a moderate decrease in the availability of chromosomal proteins for arginylation in SV40 virus-transformed cells, and this effect was not modulated by aging phenomena.

The lifespan of human diploid fibroblasts in culture can be divided into three distinct phases [1]. An initial lag phase (Phase I) is evident immediately following tissue explantation, followed by a period of rapid exponential growth (Phase II) which, in turn, is followed by a period of decreasing cell growth and viability (phase III) characterized by gross morphological and biochemical alterations. Although cultures in Phase III are defined as being senescent, the changes that produce such cultures may occur well before Phase III is reached.

Hydrocortisone has been implicated as a modulator of cellular senescence *in vitro* [2, 3]. This effect does not appear to be related to stabilization of lysosomal membranes, increased plating efficiency, or an increased fraction of the cells adhering to the culture surface. The lifespan of the fibroblasts was extended 30–40% by the continuous inclusion of hydrocortisone in the media, and the magnitude of the effect was dependent upon the relative age of the culture when initially exposed to the hormone. This modulation of aging by hydrocortisone provides a model of the study of Phase III phenomena.

An amino-terminal modification system has been isolated from bacteria, which transfers either leucine or phenylalanine to the NH_2-terminus of acceptor proteins containing lysine, arginine, and to a lesser extent histidine at that position [4, 5], and, from eucaryotic systems, arginyl-tRNA transferase has been isolated which transfers arginine to acceptor proteins with NH_2-terminal aspartic acid, glutamic acid and, to a lesser extent, cysteine [5, 6].

Arginyl-tRNA transferase has also been shown to modify erythrocyte membrane proteins [7] and chromosomal proteins *in vitro* [8]. Both of these results lend credence to the supposition that arginyl-tRNA transferase may be involved in the regulatory mechanisms of the cell. Introduction of an arginine residue into a membrane protein could modulate its response to environmental or other extracellular stimuli. The modification of chromosomal proteins was limited to the non-histone fraction of chromatin, whose function is thought to be in the regulation of specific genes.

Studies on the relationship between arginyl-tRNA transferase and cellular proliferative activity [9] indicated that the activity in regenerating rat liver was two to three times greater than that observed in control liver, and that in ascites tumor cells it was five to ten times greater than regenerating liver. On the other hand, the activity of arginyl-tRNA transferase and the ability of chromosomal proteins to accept arginine at the NH_2-terminal end diminished progressively during cellular senescence. Furthermore, these activities in SV40 transformed cell extracts remained fixed at the time when the cells were exposed to SV40 viruses [10].

In this paper, the effects of hydrocortisone and aspirin on the modification of chromatin directed by arginyl-tRNA transferase and endogenous arginyl-tRNA transferase activity are described.

METHODS

Maintenance of cell culture. Human fibroblast strains IMR90 and WI38, and their SV40 transformed cells (AG2804, VA13A, and VA132RA), supplied by the Institute for Medical Research, Cam-

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den, NJ, and Dr. Cristofalo of the Wistar Institute, were maintained in either McCoy's or Dulbecco's modified Eagle's culture medium (DEM). The media were supplemented with 10% fetal calf serum containing 50 μg/ml streptomycin and 275 units/ml penicillin G. The cells were grown at 36° in a humidified, 5% CO_2 atmosphere on 10 cm culture dishes, and subcultivation was carried out at an 8:1 split ratio using Puck's EDTA–trypsin solution. As the cells approached confluence, the medium was aspirated, and the cells were washed twice with PBS (phosphate-buffered saline containing 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.6 mM KCl, 1.4 mM KH_2PO_4) and harvested with a rubber policeman.

Preparation of arginyl-tRNA transferase and chromatin. Cells were homogenized and crude arginyl-tRNA transferase was prepared from the post-microsomal supernatant fraction as described elsewhere [11]. Nuclei obtained by centrifuging cell homogenate at 1000 *g* were purified by suspension in 1.0 ml buffer [0.05 M Tris–HCl (pH 7.4), 0.02 M KCl, 5 mM $MgCl_2$] and centrifugation over a 0.2 M sucrose cushion in the buffer for 5 min at 2000 *g*. The above procedure was repeated two more times using buffer containing 0.5% Triton X-100. Purified nuclei were then homogenized in 2.0 ml of 10 mM Tris–HCl (pH 7.8) and layered onto 2.5 ml of 10 mM Tris–HCl (pH 7.8) containing 1.7 M sucrose. The resulting interface was gently mixed and the samples were centrifuged in a Beckman SW 50.1 rotor at 130,000 *g* for 3 hr. The chromatin pellets were then washed twice in 10 mM Tris–HCl (pH 7.8) by suspension and centrifugation at 12,000 *g* for 10 min.

Purified arginyl-tRNA transferase was prepared essentially by the method of Soffer [12], involving ammonium sulfate, pH 5.2 precipitation, carboxymethyl cellulose (CMC) and diethylaminoethyl (DEAE) chromatography, except that a 70% ammonium sulfate solution of post-microsomal supernatant fraction of calf kidney was used. Transfer RNA and [^{3}H]arginyl-tRNA were prepared as previously described [7], except that tRNA was extracted from ts-RSV-NRK cultured cells grown at 35°.

Transfer of arginine from arginyl-tRNA to chromatin or α-lactalbumin. The transfer of arginine from [^{3}H]arginyl-tRNA to chromatin acceptor proteins was carried out in a 0.05-ml reaction mixture at 37° containing 100 mM Tris–HCl (pH 7.8), 45 mM KCl, 5 mM $MgCl_2$, 50 mM β-mercaptoethanol, 3 μg of purified arginyl-tRNA transferase, various amounts of chromosomal proteins, and 10,000 cpm of [^{3}H]arginyl-tRNA. Aliquots (15 μl) were treated at various time intervals with hot trichloroacetic acid as described [13]. The same experimental procedure was used for the assay of arginyl-tRNA transferase activity except that various amounts of post-microsomal supernatant fluid instead of purified arginyl-tRNA transferase, and 30 μg of α-lactalbumin instead of chromatin, were included.

Isolation and fractionations of chromosomal proteins. A complete reaction mixture (0.60 ml) to arginylate chromosomal proteins in sufficient quantity to isolate both non-histone and histone proteins contained the following: 72 μmoles Tris–HCl (pH 7.8), 2.9 μmoles ATP (potassium salt), 5.8 μmoles phosphoenolpyruvate (sodium salt), 0.048 mg pyruvate kinase 5.8 μmoles magnesium acetate, 2.8 μmoles β-mercaptoethanol, 36 μmoles KCl, 8.0 μCi [^{3}H]arginine (sp. act. 22 Ci/mmole), 240 μg tRNA, 300 μg chromatin, 72 μg purified arginyl-tRNA transferase, and 50 μg aminoacyl-tRNA synthetase. Following a 35-min incubation at 37°, the reaction mixture was centrifuged at 12,000 *g* for 20 min at 4°.

Fractionation of chromosomal proteins into histone and non-histone components was accomplished by modification of the methods of Panyim and Chalkley [14], Bhorjee and Pederson [15], Krause and Stein [16], and Kay and Singer [17]. The chromatin pelleted from the previous step was treated with 1.0 ml of 0.25 M H_2SO_4 for 30 min to induce the release of histones. The solution was centrifuged for 10 min at 12,000 *g* and the pellet was re-extracted with another 1.0 ml of 0.25 M H_2SO_4 and processed as described above. The supernatant fractions were combined, and histones were precipitated with 5 vol. ethanol and pelleted by centrifugation at 12,000 *g* for 10 min. This histone pellet was washed twice with 0.5% HCl in acetone and solubilized in 10 M urea, 0.84% polyethylene glycol-6000.

The non-histone pellet remaining after H_2SO_4 extraction was washed twice with ethanol–ether (1:1, v:v), once with ether, and solubilized in a buffer containing 0.01 M sodium phosphate (pH 7.0), 1% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol by incubating at 37° for 60 min.

The supernatant fraction derived from the centrifugation of the original reaction mixture was treated with 1 vol. of 0.5 M H_2SO_4 for 30 min, and protein was precipitated by the addition of 5 vol. ethanol. The proteins within this fraction, representing soluble proteins of the reaction mixture as well as released chromosomal proteins, were washed twice with ethanol–ether, once with ether, and solubilized in 10 M urea, 0.84% PEG-6000.

RESULTS

Protein synthesis in human fibroblasts. Since both hydrocortisone and aspirin have been implicated as modulators of cellular aging or protein synthesis *in vitro*, aging IMR90 fibroblasts were treated with hydrocortisone and aspirin, as shown in Table 1. IMR90 fibroblasts treated with either hydrocortisone (10 μg/ml) or aspirin (20 μg/ml of medium) exhibited an increase in protein synthesis, as quantitated by radioactive amino acid incorporation. The younger cells (PDL, population doubling level 33) were more responsive to hydrocortisone, showing a greater incorporation than the control, compared to the older cells (PDL 50 fibroblasts). A similar age-dependent response was observed in cells treated with aspirin.

Subcellular fractionation revealed that the increase in protein synthesis mediated by hydrocortisone was evident in nuclear, mitochondrial, post-mitochondrial supernatants, and ribosomal fractions, whereas the aspirin-mediated increase in protein synthesis was present in post-nuclear,

Table 1. Effects of hydrocortisone and aspirin on the incorporation of amino acid by aging IMR90 fibroblasts*

Subcellular fraction	Relative age (PDL)	Incorporated radioactivity: Hydrocortisone treated (10 μg/ml) (% of control)	Aspirin treated (20 μg/ml) (% of control)
Cell homogenate	33	125 ± 15† (4)	138 ± 5‡ (3)
	50	108 ± 4§ (2)	112 ± 1‖ (2)
Nuclei	33	136 ± 25‡ (3)	99 ± 11 (4)
	50	114 ± 4§ (2)	114 ± 30 (2)
Post-nuclear supernatant	33	108 ± 9 (3)	135 ± 14§ (3)
	50	103 (1)	106 (1)
Mitochondria	33	108 ± 6‡ (3)	103 ± 21 (3)
	50	112 (1)	110 (1)
Post-mitochondrial supernatant	33	154 ± 5‖ (3)	141 ± 19§ (3)
	50	106 ± 4‡ (2)	113 ± 5§ (2)
Ribosomes	33	151 ± 2‖ (2)	116 ± 21 (3)
Post-ribosomal supernatant	33	95 ± 16 (3)	111 ± 5§ (3)
	50	97 (1)	91 (1)

* IMR90 fibroblasts were incubated for 21 hr at 37° in 5.0 ml DEM containing 1.0 μCi/ml of [^{3}H]leucine or [^{35}S]methionine. Subcellular fractionation and incorporated radioactivity were determined as described in Methods. PDL indicates population doubling level. Numbers in parentheses indicate the number of samples. Mean experimental values ± one standard deviation are expressed.

† $P < 0.02$, when compared with control by Student's t-test.
‡ $P < 0.05$, when compared with control by Student's t-test.
§ $P < 0.01$, when compared with control by Student's t-test.
‖ $P < 0.001$, when compared with control by Student's t-test.

post-mitochrondrial, and post-ribosomal supernatant fractions. Furthermore, age-dependent alterations in protein synthesis were observed in some subcellular fractions, but not in the aspirin-treated nuclei and mitochondria and the hydrocortisone-treated post-nuclear, and mitochondrial, and post-ribosomal supernatant fractions.

In contrast to normal diploid cells, the SV40 transformed cell lines of IMR90 fibroblasts exhibited a decrease in protein synthesis with hydrocortisone at a concentration of 10 μg/ml and with aspirin at a concentration of 20 μg/ml, as shown in Table 2. These transformed cells do not exhibit a finite lifespan. The hydrocortisone-mediated decrease was observed in both the post-mitochondrial supernatant and ribosomal fractions, whereas an aspirin effect was present in the nuclear fraction (increase), and in the post-nuclear supernatant, post-mitochondrial supernatant

Table 2. Response of SV40 transformed IMR90 fibroblasts (AG2804) to hydrocortisone and aspirin*

Subcellular fraction	Incorporated radioactivity: Hydrocortisone treated (10 μg/ml) (% of control)	Aspirin treated (20 μg/ml) (% of control)
Cell homogenate	75 ± 9† (3)	75 ± 9‡ (3)
Nuclei	106 ± 15 (3)	122 ± 15† (3)
Post-nuclear supernatant	95 ± 9 (3)	74 ± 15† (3)
Mitochondria	84 ± 16 (3)	115 ± 21 (2)
Post-mitochondrial supernatant	66 ± 5† (3)	75 ± 3† (3)
Ribosomes	91 ± 5‡ (4)	67 ± 4† (3)
Post-ribosomal supernatant	104 ± 16 (3)	90 ± 20 (3)

* AG2804 fibroblasts were incubated for 21 hr at 37° in 5.0 ml DEM containing 1.0 μCi/ml of [^{3}H]leucine or [^{35}S]methionine. Subcellular fractionation and incorporation radioactivity were determined as described in Methods. Mean experimental values ± one standard deviation are expressed. Numbers in parentheses indicate the number of samples.

† $P < 0.05$.
‡ $P < 0.01$.

and ribosomal fractions (decrease). Although data are not shown here, hydrocortisone and aspirin stimulated protein synthesis of WI38 human fibroblasts but they inhibited that of SV40 transformants of WI38 human fibroblasts such as VA13A and VA132RA.

Effects of hydrocortisone and aspirin on arginyl-tRNA transferase activity. To determine whether a relation exists between arginyl-tRNA transferase and the alterations in protein synthesis mediated by hydrocortisone and aspirin, the activity of arginyl-tRNA transferase was assayed in IMR90 fibroblasts of PDL 33, 49, 60 and SV40 transformed IMR90 cells (data not shown). The results of these experiments indicated that, despite a change in total cellular protein content induced by hydrocortisone and aspirin, the degree of transferase activity remained constant at a particular PDL. Furthermore, concentrations of hydrocortisone ranging from 2×10^{-4} M to 1×10^{-5} M and of aspirin from 1×10^{-3} M to 5×10^{-5} M had no significant effects on arginyl-tRNA transferase activity when a transfer of arginine from arginyl-tRNA assay system was used as described in Methods. These data suggest that any alterations in transferase activity produced *in vivo* are not the result of direct stimulation or inhibition of the enzyme.

Chromatin acceptor capacity in aging IMR90 fibroblasts. The ability of chromosomal proteins to accept arginine via arginyl-tRNA transferase was examined with regard to cellular aging and hydrocortisone-induced modulation of aging. A considerable decline in the chromatin acceptor capacity was observed between normal cells of PDL 30 and 40 (Fig. 1). The extent of protein modification was dependent upon the quantity of chromosomal proteins added. The relationship observed was not directly proportionate, perhaps due to substrate saturation of the exogenously added arginyl-tRNA transferase.

Addition of hydrocortisone to the growth medium at a concentration of 10 μg/ml produced a dramatic increase in the availability of chromosomal proteins for modification (Fig. 1). The magnitude of stimulation by hydrocortisone was inversely proportional to the age of the culture, resulting in an increase in chromatin acceptor capacity of PDL 30 cells and only a slight increase in PDL 70 cells. Both treated and untreated cells exhibited age-dependent decreases in chromatin acceptor capacity, although the level of modification for hydrocortisone-treated fibroblasts was quantitatively higher. When the results of all hydrocortisone experiments were expressed as a percent of control at each age level, cells at the relative age of PDL 30 exhibited chromatin acceptor capacities that were much higher than control, whereas PDL 70 cells were only slightly above control when treated with hydrocortisone. This increase in chromosomal proteins available for modification could be the result of either hydrocortisone-induced protein synthesis or conformation alterations in pre-existing chromosomal proteins.

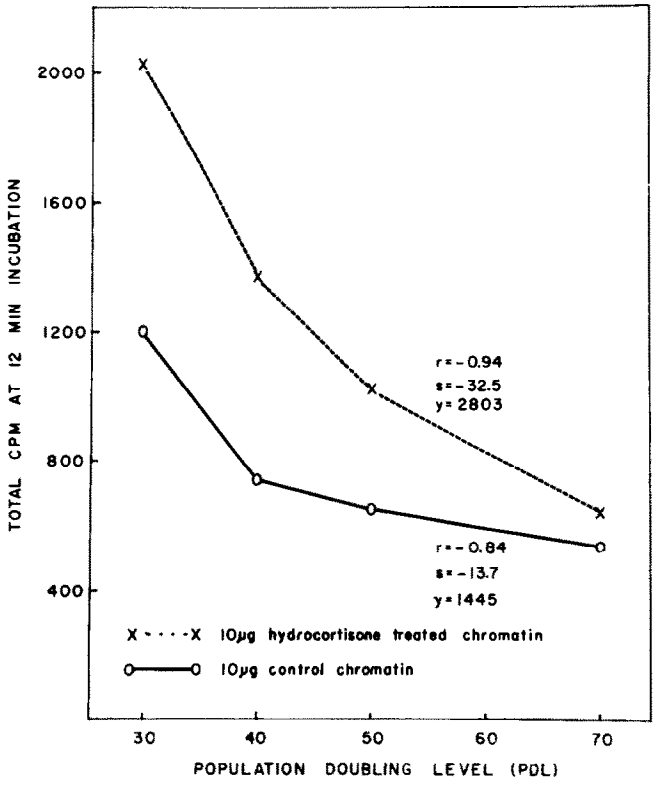

Fig. 1. Chromatin acceptor capacity in hydrocortisone-treated IMR90 fibroblasts. [^{3}H]Arginyl-tRNA assay system, as described in Methods, contained 10 μg of chromatin obtained from cells grown with or without hydrocortisone. Each value is the mean of triplicate analyses.

Table 3. Fractionation of chromosomal proteins modified by arginyl-tRNA transferase *in vitro*—effect of aging and hydrocortisone*

Cell type	Histones cpm	Histones %	Non-histones cpm	Non-histones %	Released proteins cpm	Released proteins %
IMR90 PDL 38	699	100	1813	100	60,549	100
IMR90 PDL 38 + HC†	787	113	1351	69	81,044	134
IMR90 PDL 53	469	67	1799	99	24,524	41
IMR90 PDL 53 + HC†	494	71	1334	74	29,876	48

* Chromatin derived from various cell strains was incubated in a reaction mixture, as described in Methods, for 35 min at 37° and subjected to fractionation into histones, non-histones, and released chromosomal protein fractions. In all cases, more than 90% of incorporated radioactivity was recovered in the released-protein fraction (supernatant fraction).

† HC indicates hydrocortisone.

When larger quantities of chromatin were subjected to modification, as described in Methods, and were fractionated into histone, non-histone, and released chromosomal protein fractions, the results shown in Table 3 were obtained. Regardless of the age of the cells or pretreatment with hydrocortisone, the major portion of the radioactivity incorporated into chromatin was isolated in the supernatant fraction of the reaction mixture, suggesting that the chromosomal proteins were modified and then released from the chromatin, or that modification occurred subsequent to release. As was observed with intact chromatin, the incorporation of arginine into the released chromosomal protein fraction exhibited an age-dependent decrease, as well as a marked increase when the cells were pretreated with hydrocortisone. Hydrocortisone produced little difference between incorporation into the histone fraction when compared to control, although the age-related effect was apparent. In the non-histone fraction, hydrocortisone induced a greater release from the treated cells, although there was no significant difference between the "young" and "old" chromatin.

In contrast to normal IMR90 fibroblasts, cells transformed by SV40 viruses exhibited a decrease in chromatin acceptor capacity when treated with hydrocortisone (Table 4). The effect was the same whether 10 μg or 20 μg of chromatin was added to the reaction mixture, and the acceptor capacity remained constant regardless of the number of subcultivations that were performed. Therefore, SV40-transformed fibroblasts are not subject to the age-dependent decreases in acceptor capacity that are evident in normal diploid fibroblasts. Similar results were obtained with WI38 human fibroblasts and their SV40 transformants.

DISCUSSION

Human fibroblasts in culture provide a unique model for the study of cellular senescence, as Hayflick first proposed in 1965 [1]. They have a reproducibly finite lifespan that is measured in periods of months, not years; the microenvironment can be controlled very precisely; the addition of hydrocortisone to the culture medium can extend the lifespan of the fibroblasts by 30–40%; and the problem of interspecies comparison is eliminated. The data presented here show that hydrocortisone mediated an increase in amino acid incorporation that was evident in the post-mitochondrial supernatant, ribosomal, and nuclear fractions, and that the magnitude of the response was dependent upon the relative age of the cells. These findings are consistent with the mechanism of action of hydrocortisone, which is thought to involve a cytoplasmic receptor for the stimulation of specific mRNA synthesis [18]. Specific induction of mRNA synthesis is observed even if the overall cellular effect is one of inhibition, as is the case with

Table 4. Effect of hydrocortisone on the chromatin acceptor capacity of SV40 transformed fibroblasts*

Incubation time (min)	Chromatin acceptor capacity (cpm) Control 10 μg	Control 20 μg	Hydrocortisone treated (10 μg/ml) 10 μg	Hydrocortisone treated (10 μg/ml) 20 μg
4	261	337	208	257
8	383	590	354	381
12	489	659	380†	518†

* [^{3}H]Arginyl-tRNA assay systems, as described in Methods, contained either 10 μg or 20 μg of chromatin derived from the indicated experimental groups. Each value is the mean of duplicate analysis. Three such experiments were performed on the chromatin isolated from SV40 virus transformed fibroblasts (AG2804).

† $P < 0.01$.

various transformed cell lines [19]. Therefore, the effect of hydrocortisone was observed in the nuclear as well as the cytoplasmic compartment, whereas aspirin, whose effects are presumably mediated via another mechanism [20] and which does not modulate cellular senescence, exhibited only cytoplasmic increases in protein synthesis. In contrast to normal diploid cells, the transformed cell lines showed a decrease in protein synthesis when treated with hydrocortisone or aspirin. The hydrocortisone response was inhibitory, even though the effect is thought to be mediated by the synthesis of specific mRNA.

Evaluations of the relationship between hydrocortisone-induced modulation of cellular senescence and arginyl-tRNA transferase indicated that neither hydrocortisone nor aspirin had a direct effect on transferase activity *in vitro*; furthermore, although both hydrocortisone and aspirin produced an *in vivo* stimulation of protein synthesis, the relative degree of transferase activity remained constant. Therefore, no measurable increase in transferase activity was observed when control cells and hydrocortisone-treated cells were compared on a per microgram of protein basis. The most striking effect of hydrocortisone was its stimulation of chromosomal protein acceptor capacity. Normal fibroblasts exhibited a considerable decrease in chromatin acceptor capacity as a consequence of aging, and this effect was also apparent in cells treated with hydrocortisone at a quantitatively higher level. Specifically, hydrocortisone-treated cells had chromatin acceptor capacities that were significantly higher than the control values, depending upon the relative age of the cells. In other words, the younger cells were more sensitive to hydrocortisone-induced stimulation of chromatin modification. This sensitivity correlates quite well with the ability of hydrocortisone to extend the lifespan of human fibroblast cultures [2], which is also dependent upon the age of the cultures when first exposed to the hormone. Although the mechanism of action is not clear, the increase in the availability of chromosomal acceptor proteins could be the result of either stimulation of nuclear protein synthesis or hydrocortisone-induced alteration in the conformation of some existing chromosomal proteins.

Fractionation of the chromatin into histone, non-histone and released chromosomal protein fractions following modification by arginyl-tRNA transferase provided data that are consistent with the premise that the enzyme may be involved with cellular regulatory functions. The modification of proteins could alter the association of the proteins with the chromatin, which in turn could alter the transcriptional capacity of the cell. These differences are reflected in the quantity of modified proteins isolated in the released chromosomal protein fractions and are independent of the relative age of the cells, as well as the treatment with hydrocortisone.

Hydrocortisone had the opposite effect on fibroblasts transformed by an SV40 virus, causing a decrease in chromatin acceptor capacity. The level of acceptor capacity in both the control and hydrocortisone-treated SV40 fibroblasts remained the same throughout numerous subcultivations, indicating that these cells were not subject to the age-associated decline in chromatin acceptor capacity and, in fact, were refractory to aging phenomena in general. These results have led to the conclusion that arginyl-tRNA transferase-directed modification of chromosomal proteins is a marker of cellular senescence for the following reasons: (1) a significant decrease in chromatin in acceptor capacity was observed during cellular senescence, (2) hydrocortisone, which can extend the lifespan of cultured fibroblasts, induced an increase in acceptor capacity concomitant with this extended lifespan, and (3) the acceptor capacity of SV40 transformed fibroblasts, which do not show aging phenomena, did not change, even following repeated subcultivation.

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