Experimental Gerontology, Vol. 34, No. 1, pp. 35–45, 1999

0531-5565/99 $–see front matter

ELSEVIER

PII S0531-5565(98)00056-4

FURTHER EVIDENCE FOR THE REJUVENATING EFFECTS OF THE DIPEPTIDE L-CARNOSINE ON CULTURED HUMAN DIPLOID FIBROBLASTS

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Abstract—We have confirmed and extended previous results on the beneficial effects of L-carnosine on growth, morphology, and longevity of cultured human fibroblasts, strains MRC-5 and HFF-1. We have shown that late-passage HFF-1 cells retain a juvenile appearance in medium containing 50 mM carnosine, and revert to a senescent phenotype when carnosine is removed. Switching cells between medium with and without carnosine also switches their phenotype from senescent to juvenile, and the reverse. The exact calculation of fibroblast lifespans in population doublings (PDs) depends on the proportion of inoculated cells that attach to their substrate and the final yield of cells in each subculture. We have shown that carnosine does not affect cell attachment, but does increase longevity in PDs. However, the plating efficiency of MRC-5 cells seeded at low density is strongly increased in young and senescent cells by carnosine, as shown by the growth of individual colonies. We have also demonstrated that very late-passage MRC-5 cells (with weekly change of medium without subculture) remain attached to their substrate much longer in medium containing carnosine in comparison to control cultures, and also retain a much more normal phenotype. Carnosine is a naturally occurring dipeptide present at high concentration in a range of human tissues. We suggest it has an important role in cellular homeostasis and maintenance.

Key Words: human fibroblasts, senescence, rejuvenation, carnosine

INTRODUCTION

L-CARNOSINE IS A DIPEPTIDE consisting of β-alanine linked at its carboxyl terminus to the amino group of L-histidine (β-alanyl-L-histidine). It is synthesized by the enzyme carnosine synthetase, and broken down by carnosinase. It is widely distributed in tissues, and is present at a particularly high concentration in skeletal muscle and the olfactory lobe of the brain. Carnosine has a number of important properties, including antioxidant activity, ability to chelate divalent

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(*Received* 12 *May* 1998; *Accepted* 26 *August* 1998)

cations such as copper, neutralisation of acids (such as lactic acid), and the inhibition of nonenzymic glycosylation of proteins (Quinn *et al*., 1992; Hipkiss *et al*., 1995). This inhibition is due to its ability to react with keto- or aldehyde groups in sugars through the Amadori and Maillard reactions (Monnier, 1988). It may, therefore, remove active sugars that might otherwise react with the terminal amino group of lysine in proteins.

We previously showed that human diploid fibroblasts grow with a normal population doubling rate in standard media containing physiological concentrations of carnosine, such as 20 mM (McFarland and Holliday, 1994). Moreover, these cells had an extended lifespan, both in population doublings and chronological time. Although these cells still had a finite lifespan, their appearance at the end of proliferation was far more like that of young fibroblasts than senescent untreated cells. These senescent cells become granular, are irregular in size and shape, and debris accumulates in the medium, as cells detach from the substratum. We also showed that senescent cells in normal medium transferred to medium containing carnosine were rejuvenated in their morphological appearance and, at least in some cases, continued to proliferate. Transfer of these rejuvenated cells back to normal medium without carnosine lead to the appearance of the senescent phenotype after a few days. Finally, experiments with the limited quantity of D-carnosine that was available showed that this isomer was inactive. In another study we showed that L-carnosine can inhibit the growth of transformed human or rodent cells, whereas the D-isomer was inactive (Holliday and McFarland, 1996). These results strongly indicate that the effects of L-carnosine are not due solely to its chemical properties, but that it plays an active role in cellular metabolism. We have now repeated and extended the earlier experiments, and detected some important additional beneficial effects of L-carnosine.

MATERIALS AND METHODS

Cell strains

The two human diploid fibroblast strains are the same as previously used, namely, fetal lung strain MRC-5 and foreskin cells strain HFF-1 (McFarland and Holliday, 1994).

Cell culture

Cells were grown in Dulbecco's modification of Eagles' minimum essential medium (DMEM Gibco BRL Cat. No. 12800-058) containing 0.45% glucose, or in MEM (Gibco BRL Cat. No. 61100-053) containing 0.1% glucose. The MEM was supplemented with 0.1% sodium pyruvate (which is present in DMEM; see Holliday and McFarland, 1996). Both media contained 10% fetal calf serum, penicillin (60 μg/mL), streptomycin (100 μg/mL), and nonessential amino acids (Cytosystems). Cells were grown in 25 cm^2 flasks or 10 cm dishes at 37°C with 5% CO_2. Cells were harvested with trypsin/versene, dispersed, and counted with a model ZF6 Coulter Counter. Cells were split in ratios of 1:2, 1:4, or 1:8. Population doublings (PDs) were calculated from the ratio of the final yield of cells to the number inoculated which attach to the substrate. This provides an accurate measure of cell growth, but the split ratios 1:2, 1:4, and 1:8 correspond to one, two, and three passages, which are broadly equivalent to population doublings. (Passage levels are important when cells are not counted, as in cases where cells were switched from normal medium to that containing L-carnosine, or vice versa). The medium was changed weekly if cells were not confluent or when confluent in some experimental studies (see Results). When cells were grown in plates, the medium was changed weekly and the colonies stained with Giemsa after three weeks to one month.

L-carnosine

L-Carnosine (99% purity) was obtained from the Sigma Chemical Company. Stock solutions of 100 mM in DMEM or MEM were prepared, filter sterilized, and diluted to 20 mM, 30 mM, or 50 mM in these growth media. L-Carnosine is referred to as carnosine hereafter.

Photomicroscopy

A Nikon Diaphot inverted phase-contrast microscope with a Nikon HFX Camera attachment and Kodak technical pan film were used.

RESULTS

Rejuvenation of senescent cells by L-carnosine

We previously showed that MRC-5 cells grown in 20 mM or 30 mM had an extended lifespan and retained a normal morphology for a longer period than cells grown in unsupplemented medium. This basic observation is illustrated in Fig. 1, which shows confluent early-passage MRC-5 cells, cells grown to late passage in normal DMEM and cells grown continuously in DMEM supplemented with 20 mM carnosine to a later passage than the control culture.

HFF-1 cells grow in a higher concentration of carnosine than MRC-5 cells. In 50 mM carnosine growth is quite slow, and a high PD level is not achieved. It is striking that these cells retain a normal juvenile appearance even when growth ceases (see Fig. 8, McFarland and Holliday, 1994). We have consistently found that senescent HFF-1 cells grown in normal medium were restored to a rejuvenated phenotype when transferred to DMEM containing 50 mM carnosine. This occurs within seven days if the cells are split to carnosine medium. However, if the medium is just changed to DMEM containing 50 mM carnosine, it takes up to 21 days for senescent cells to become rejuvenated. We have also consistently observed the reverse effect, that is, the change from a juvenile to a senescent phenotype after removal of carnosine. This result is illustrated in Fig. 2. In addition, we have switched late-passage HFF-1 cells several times between normal DMEM and DMEM supplemented with 50 mM carnosine, and in all cases the phenotype switched from senescent to normal, or the reverse. Typical results are illustrated in Fig. 3. In all cases the rejuvenated phenotype is completely dependent on the continued presence of the dipeptide.

Effects of carnosine on plating efficiency and growth of senescent cells

It is well known that vigorous growth of human fibroblasts is dependent on seeding density. Below a given cell density cell division is inhibited, but the actual "plating efficiency" is controversial. This is the proportion of isolated cells that will form a colony. Most laboratories, including ours, record low plating efficiencies for primary human fibroblasts without a feeder layer, but others have established procedures that give high plating efficiencies (Smith and Hayflick, 1974; Smith and Whitney, 1980). We carried out experiments to determine the effect of carnosine on plating efficiency, using young and senescent MRC-5 cell populations. 10^3 cells were seeded per plate, the medium was changed weekly, and when colonies appeared they were stained with Giemsa. Typical results are shown in Fig. 4. It is clear that 20 mM carnosine in DMEM medium has a strong stimulatory effect on colony formation of young cells. Even more striking is the effect on late-passage senescent cells. Because untreated late passage cells have very little remaining growth potential, one would expect little colony growth, as is the case. However, the presence of carnosine clearly rejuvenates these cells, allowing more colonies to

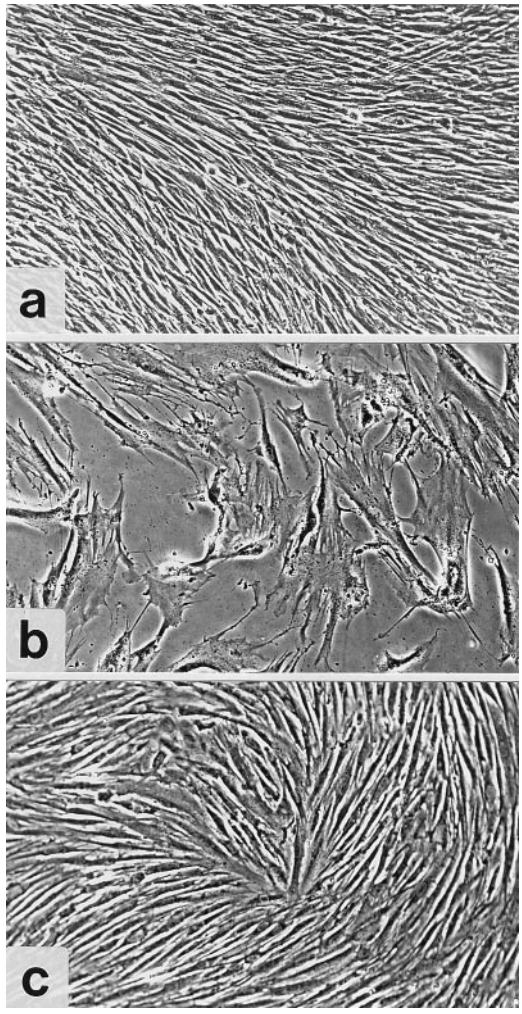

FIG. 1. The response of MRC-5 cells to long-term growth in 20 mM carnosine in DMEM medium: (a) young confluent cells at passage 14; (b) untreated cells at PD 55.1 demonstrating a senescent phenotype; (c) cells grown in DMEM supplemented with 20 mM carnosine (from passage 14) showing a nonsenescent phenotype at PD 60.6.

appear and grow to a larger size. The largest colonies are equivalent to about 15 PDs from a single cell.

Attachment of trypsinized cells to their substrate in relation to longevity

In the calculation of the population doublings (PD), it is normal to count the cells harvested after trypsin treatment and assume that the fraction of cells added to a fresh flask achieve 100% attachment. Therefore, the ratio of the inoculated cells and the final yield provides the PD increment for that individual passage. Any factor influencing the extent of cell attachment will also affect the calculated PD increment. It is, therefore, possible that the effect of carnosine in increasing longevity in PDs might also be related to an effect on cell attachment.

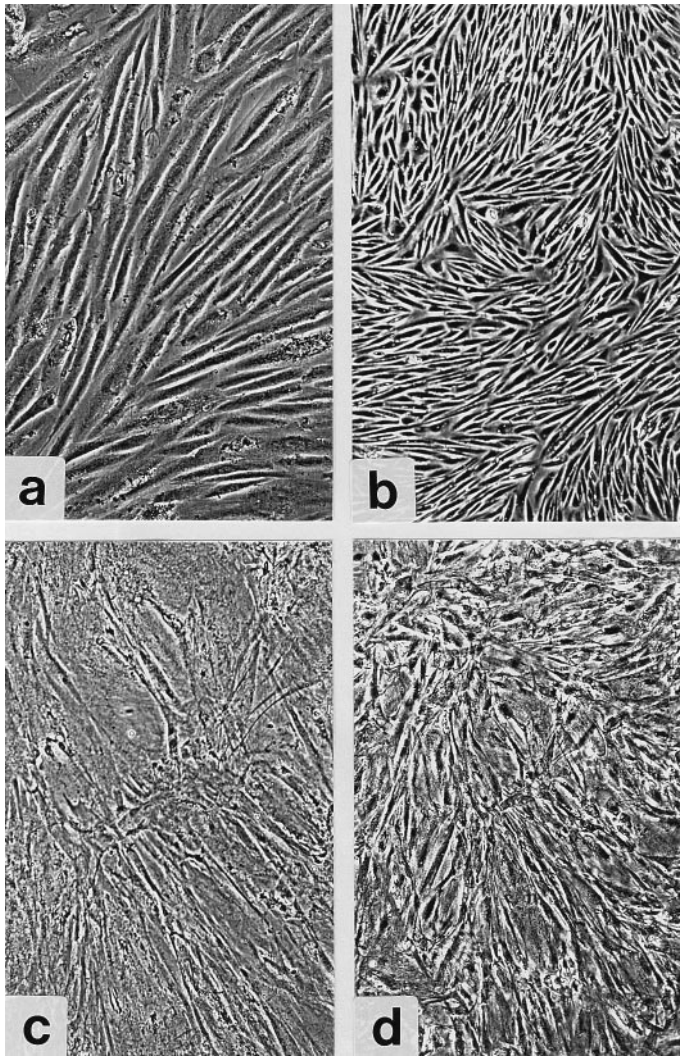

FIG. 2. HFF-1 cells grown in DMEM supplemented with carnosine, and the effect of removing carnosine: (a) cells at passage 45 grown 12 weeks in 50 mM carnosine; (b) the same at low power; (c) 30 days after subculture (1:2 split) in DMEM with weekly changes of medium; (d) the same at low power.

We, therefore, carried out experiments in which an additional flask was seeded with the same number of cells as the ongoing growing population. After the cells had fully attached, but before any subsequent growth (17–20 h), this additional flask was trypsinized and the cells counted. This provides a measure of cell attachment, and also a more accurate measure of the PD increment for that passage. The proportion of cells attached was lower than we expected for control young fibroblasts, and this proportion was largely unaffected by the presence of carnosine. Two experiments were carried out with MRC-5 cells: one with unsupplemented DMEM and DMEM with 20 mM and 30 mM carnosine, and the other with MEM containing

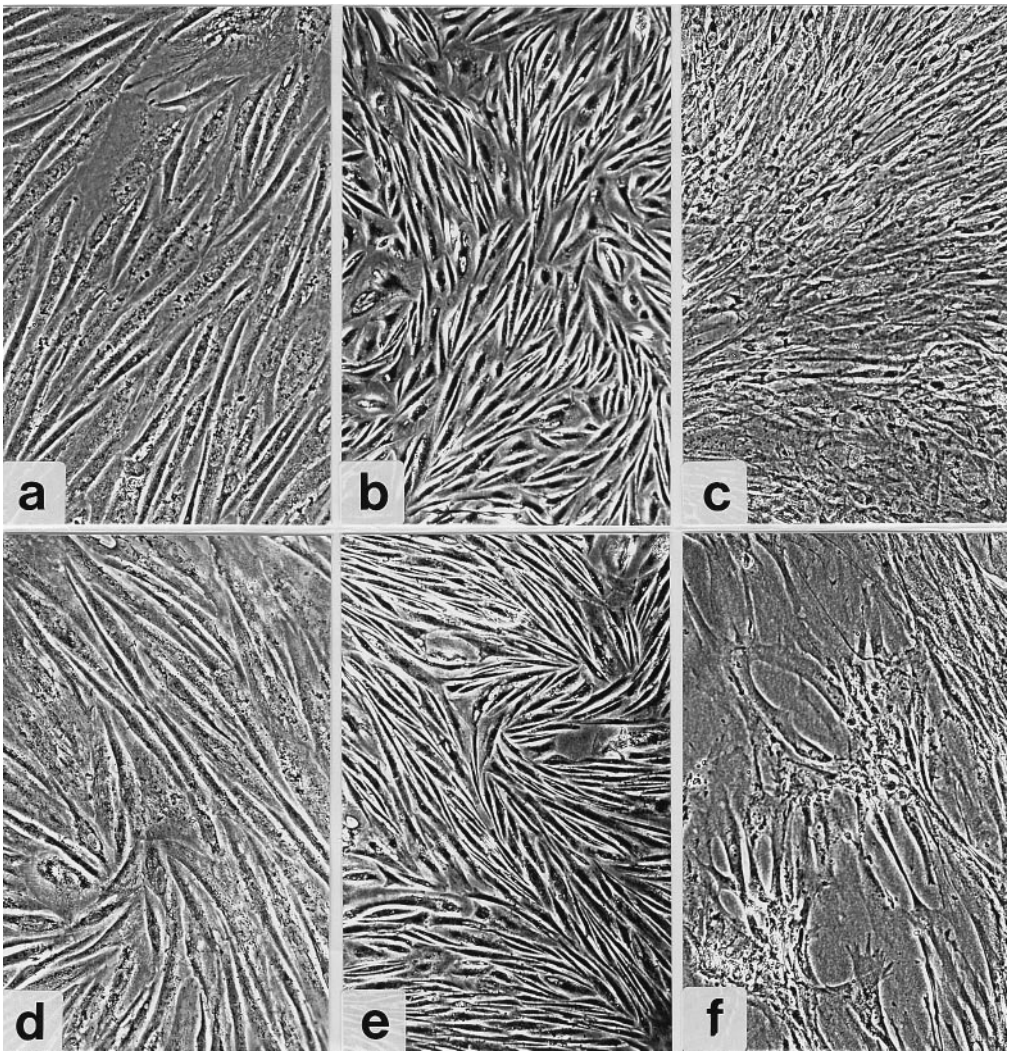

FIG. 3. HFF-1 cells switched from DMEM supplemented with carnosine to unsupplemented medium, then to carnosine supplemented medium, and finally back to unsupplemented DMEM: (a) cells at passage 35 grown from passage 30 in DMEM containing 50 mM carnosine (over a period of 182 days with five 1:2 splits); (b) the same, at low power to show normal whorls of growth; (c) 43 days after transfer to normal DMEM (passage 37 cells from two 1:2 splits and weekly medium changes); (d) 34 days after return to DMEM supplemented with 50 mM carnosine (passage 38 cells from one 1:2 split and weekly medium changes); (e) the same at low power; (f) 48 days after return to normal DMEM (passage 39 cells from one 1:2 split and weekly medium changes). These cells did not proliferate further.

1 mM sodium pyruvate, with or without 20 mM carnosine. The results are shown in Table 1. In Experiment 1 the lifespan was increased 11.4 PDs by 20 mM carnosine and 7.2 PDs by 30 mM carnosine. In Experiment 2 the lifespan was increased 10.8 PDs by 20 mM carnosine. Carnosine also had an effect on the chronological age of the cultures, particularly in DMEM

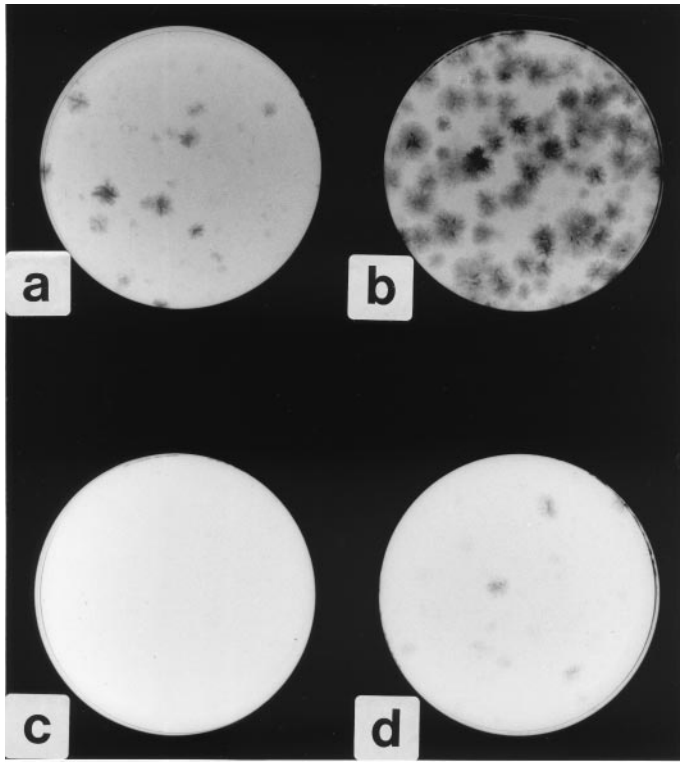

FIG. 4. Colonies produced from plating 10^3 MRC-5 cells in DMEM with or without carnosine: (a) passage 28 cells in DMEM; (b) the same cells in DMEM supplemented with 20 mM carnosine; (c) passage 56 cells in DMEM; (d) the same cells in DMEM supplemented with 20 mM carnosine. The early-passage cells were grown 22 days (four changes of medium) before staining with Giemsa. The late-passage cells were grown 31 days (five changes of medium) before staining.

medium. Control cultures (starting at PD level 20) normally cease proliferation after 120–140 days, assuming the cells are passaged when they reach confluence. However, the 20-mM and 30-mM cultures in DMEM continued to grow slowly for at least twice as long. The last subcultures were 315 days (20 mM) and 321 days (30 mM) after the beginning of the experiment. In these experiments only about two-thirds of the cells on average attached at each passage, but the actual level was quite variable, as the standard derivations show. The longevity results obtained are similar to those previously published, and show that the effect of carnosine is in no way related to the proportion of attached cells.

The attachment of late-passage senescent cells in the presence or absence of carnosine

We examined the attachment of MRC-5 cells in late-passage cultures over a long period in normal DMEM and DMEM containing 30 mM carnosine. A number of parallel senescent control cultures in 25 cm^2 flasks were accumulated. In each case the number of cells seeded in the flask was recorded, the average number being 9.6×10^5. The medium was changed weekly, and at intervals a culture was trypsinized and counted. The percent increase or decrease in cell number in relation to chronological age over a 133-day period was obtained. These results are

TABLE 1. THE LIFESPANS OF MRC-5 POPULATIONS IN PDs, BASED ON THE ACTUAL NUMBER OF CELLS THAT REATTACHED AFTER TRYPSINISATION

Experiment No.	*Medium*	*Carnosine*	*Final PD level*	*No. of subcultures*	*% Cell attachment ± SD*
1	DMEM	none	59.8	13*	73.6 ± 17.6
	DMEM	20 mM	71.2	24	72.1 ± 20.7
	DMEM	30 mM	67.0	22	63.2 ± 21.5
2	MEM	none	65.5	13*†	68.2 ± 14.1
	MEM	20 mM	76.3	16*	64.4 ± 20.6

The experiments were started with cells at passage 21 and carnosine was added after one 1:8 split. Both DMEM and MEM contained 1 mM Na pyruvate. SD = standard deviation.
*Last subculture (<25% attachment) is not included in the values in the last column.
†Attachment not determined in two subcultures (average value used to calculate PD increment).

summarized in Table 2. A parallel set of cultures was transferred to 30 mM carnosine at passage 48 and then grown to passage 60 with the accumulation of multiple parallel cultures. Although these cells were significantly less senescent in appearance, the average number of cells seeded per flask was 6.3×10^5, i.e., lower than the controls. The results of this part of the experiment are also summarized in Table 2. The comparison of untreated and treated cells allows the following conclusions. 1) Initially, the control senescent cells increased slightly in numbers, showing that some residual growth could occur. 2) During the period 6 to 13 weeks, the number of cells declined to 71% of the initial number, and from 14 to 19 weeks to 25%. 3) The cells in carnosine, which were at a later passage level, grow to a greater extent over the first eight weeks. 4) During the period 9 to 17 weeks the number of cells was still higher than the number seeded per flask, and this number only declined over the final period of 18–21 weeks. It is clear that carnosine promotes the attachment and survival of cells over a long period. The result is particularly significant because the control cells were at an earlier passage level that the treated cells. The cells held in medium containing carnosine for a long period also had a much more normal phenotype than those in unsupplemented medium. This is illustrated in Fig. 5.

TABLE 2. THE ATTACHMENT OF MRC-5 CELLS IN PARALLEL LATE-PASSAGE CULTURES IN 25 cm^2 FLASKS IN THE PRESENCE AND ABSENCE OF 30 mM CARNOSINE

Treatment	*Passage level (average cells seeded)*	*No. weekly changes of medium*	*No. cultures*	*Average no. cells harvested (% of initial seeded)*
Control, DMEM	56	3–4	2	136
	(9.50×10^5)	5–12	6	71
		13–18	5	25
		(133 days)		
DMEM+	60	5–7	3	234
30 mM	(6.3×10^5)	8–16	9	145
carnosine		17–20	4	70
		(147 days)		

There were no control cell counts at weeks 7, 8, and 16. Treated cells were grown in 30 mM carnosine from passage 48.

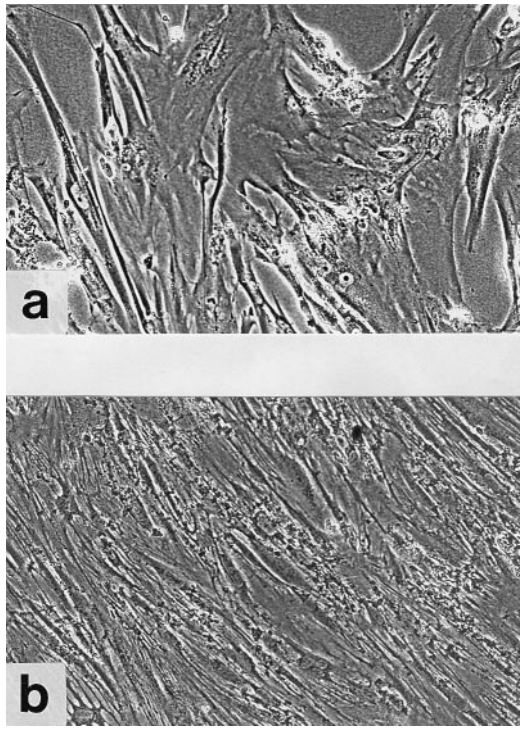

FIG. 5. MRC-5 cells from the experiment documented in Table 2: (a) untreated cells at passage 56, incubated for 84 days with weekly changes of medium; (b) cells grown in DMEM supplemented with 30 mM carnosine at passage 60 incubated for 127 days with weekly changes of the same medium.

DISCUSSION

We have carried out further experiments on the effects of carnosine on the growth, morphology, and longevity of MRC-5 and HFF-1 human fibroblasts. In general, the results fully confirm the earlier experiments and have also provided new information. We have carefully examined the effects of high concentrations of carnosine (50 mM) in rejuvenating senescent populations grown in normal DMEM medium. Senescent cells fail to line up in the normal parallel arrays of young fibroblasts, and have an irregular granular appearance. Transfer of these senescent populations to 50 mM carnosine, or changing the medium to 50 mM carnosine, had a dramatic effect in restoring the phenotypic characteristics of young HFF-1 fibroblasts. These cells have the characteristic "whorls" of young fibroblast cultures, and the cells are far more uniform in their appearance. Also, transfer of these rejuvenated cells from DMEM containing 50 mM carnosine to unsupplemented medium resulted in the reappearance of the senescent phenotype (Fig. 2 and Fig. 3).

We have used MRC-5 to examine the effects of carnosine on cell attachment and plating efficiency. First, we demonstrated that carnosine significantly improved the plating efficiency, that is, the number of seeded cells that grow to form colonies in dishes (Fig. 4). Second, it has been shown that the increased longevity of MRC-5 cells grown in the presence of carnosine is in no way related to an effect of the dipeptide on the proportion of cells that attach to the substrate after the flasks are inoculated (Table 1). We did this because the lower the percentage

of cells attached, the higher would be the calculated PD values. Third, it was demonstrated that late-passage cells grown and then held in medium containing 30 mM carnosine remained attached to the plastic substrate for a longer period than control cells, and also retained a more normal phenotype (Table 2 and Fig. 5).

In a separate study we showed that cell growth in the presence of carnosine was strongly influenced by the availability of pyruvate (Holliday and McFarland, 1996). In particular, a wide selection of neoplastic or transformed cells were killed by carnosine in the absence of pyruvate, while they grew vigorously in its presence. With normal diploid cells, pyruvate also has an influence on growth. For example, MRC-5 cells will not grow in MEM containing 30 mM carnosine, whereas they will do so in DMEM. This difference is largely due to the pyruvate in DMEM, although the higher glucose also has an effect. However, the effect of high concentrations of carnosine in normal medium lacking pyruvate is not cytostatic on normal cells, because they quickly resume growth if carnosine is removed (results not shown). On the other hand, carnosine is cytotoxic to cancer cells in the same medium. It was suggested that the sensitivity of these cells to carnosine is due to their increased dependence on glycolysis relative to respiration as a source of energy (Aisenberg, 1961; Dills, 1993). It is known that carnosine reacts strongly with glycolysis intermediates such as triose sugar phosphate (Hipkiss *et al*., 1995; A.R. Hipkiss, A. Stevens personal communication; also see Hamada *et al*., 1996). Thus, cancer cells in the presence of carnosine and absence of pyruvate would have insufficient energy for growth or survival. It is possible that the effect of carnosine in increasing the growth potential of human fibroblasts is due to its ability to react with sugars. This reaction provides the basis for carnosine's ability to competitively inhibit protein glycosylation, at least in in vitro experiments (Hipkiss *et al*., 1995). Such glycosylation leads to the formation of high molecular-weight fluorescing pigments known as advanced glycation end products, or AGEs (Monnier, 1988). It is also possible that AGEs are chemically related to the autofluorescent material that is known to accumulate during the lifespan of human fibroblasts (Rattan *et al*., 1982).

It is remarkable that a small dipeptide such as carnosine has so many different biochemical properties (see Introduction), and we favor the view that it may have a very important role in controlling cellular homeostasis. It is important to note that the amount of carnosine in mouse skeletal muscle tissues is significantly lower than in human muscle tissue (1 mM instead of 20 mM; J. Michaelis, personal communication). It is well known that the efficiency of a number of cell maintenance mechanisms correlates with mammalian species lifespan (Holliday, 1995, 1996), and we therefore propose that carnosine is an important component of cellular maintenance mechanisms.

Strong environmental effects on fibroblast lifespan do not obviously relate to intrinsic mechanisms based on a "molecular clock," such as the loss of telomeric DNA, or DNA methylation (Matsumura *et al*., 1989; Harley, 1991; Holliday, 1995; Bodnar *et al*., 1998). However, it is certainly possible that such treatments influence the rate of the clock mechanism, perhaps by an indirect effect. Further studies are necessary to relate known molecular parameters to treatments which increase, or decrease, mammalian lifespans.

Acknowledgments—We thank Alan Hipkiss and Geoffrey Grigg for helpful discussion and encouragement.

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