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# Carnosine Sustains the Retention of Cell Morphology in Continuous Fibroblast Culture Subjected to Nutritional Insult

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L-Carnosine (β-alanyl L-histidine), occurring abundantly in skeletal muscles, has been suggested to possess antioxidant and anti-aging properties. Using three different experimental approaches (microscopic, flow cytometric and ELISA for one of the markers of DNA oxidative damage) this study on rat embryonic fibroblasts demonstrates that L-carnosine at 30 mM concentration sustains the retention of cell morphology even during a nutritional insult for five weeks. Also, L-carnosine significantly reduces the formation of 8-hydroxy deoxyguanosine (8-OH dG) in the cells after four weeks of continuous culture. Thus it could be inferred that the anti-senescent effect of L-carnosine is probably linked to its inhibition of formation of intracellular 8-OH dG during oxidative stress. 

L-Carnosine (β-alanyl histidine) is abundantly present in the skeletal muscles and specific tissues of the nervous system of vertebrates including humans (1–3). Gardner et al. (4) had presented evidence in humans that upto 14% of intact L-carnosine was recovered in the urine suggesting that intact L-carnosine crosses the intestine to an extent much greater than hitherto recognized. During the past decade, the antioxidant properties of L-carnosine have also been reported from quite a number of laboratories (5–10).

A recent report by McFarland and Holliday (11) that L-carnosine at concentration 20–50 mM, in addition to retarding senescence, also rejuvenated senescent cultures of human foreskin fibroblasts (HFF-1 strain) and fetal lung fibroblasts (MRC-strain) is of gerontological interest. This effect was found to be absent in compounds such as D-carnosine, homocarnosine, anserine and β-alanine. McFarland and Holliday (11) had suggested that though L-carnosine is an antioxidant, it probably preserves the cellular integrity by its effects on protein metabolism.

Oxidative DNA damage has been attributed as one of the main causes of cellular senescence (12) and 8-hydroxy deoxyguanosine (8-OH dG) is one of the major products of oxidative DNA damage (13). Yamamoto et al. (14) had shown that the photosensitized formation of 8-OH dG in DNA by riboflavin may be involved in photocarcinogenesis as well. In this study we demonstrate in fibroblasts that L-carnosine's proposed antisenescent effect (11) is probably linked to its inhibition of formation of 8-OH dG.

## MATERIALS AND METHODS

*Materials.* Unless otherwise stated all chemicals used in this investigation were purchased from Sigma Chemical Co. and used without further purification. L-carnosine was purchased from Wako Pure Chemicals (Tokyo). The 8-OH dG standard, synthesized according to Kasai and Nishimura (15), and monoclonal IgG specific for 8-OH dG were supplied by Toray Chemicals (Kamakura, Japan).

*Cell culture.* Fibroblasts derived from 3Y1B clone 1–6 line of embryo from Fischer rat strain (16) were grown in DMEM (Gibco BRL), supplemented with 10% fetal calf serum (FCS) and a cocktail of antibacterial-antimycotic mixture consisting

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of penicillin G sodium, streptomycin, amphotericin B and gentamicin. The DMEM did not contain the nutrient antioxidants (such as L-ascorbic acid, glutathione, lipoic acid and $\alpha$-tocopherol), guanosine and its derivative 2-deoxyguanosine. The fibroblasts were seeded at $5 \times 10^4$ cells/ml in 8 $cm^2$ petri dishes and incubated in a humidified atmosphere of 5% $CO_2$: 95% air at 37°C.

To assess the antioxidant effect of L-carnosine in fibroblasts, the cells were transferred to petri dishes containing the culture medium supplemented with either 10 mM or 30 mM L-carnosine and allowed to grow for five weeks at 37°C without any weekly change in culture medium or any additional nutritional replenishment. A negative control was also set up for comparison. The culture medium from each treatment was collected at weekly intervals and centrifuged at 1500 g for 5 min to remove any suspended cell debris. The collected supernatant was frozen at −80°C until assay.

*Photomicroscopy.* A phase contrast microscope (Olympus IX70, Japan) with a camera attachment (Olympus SC35 Type 12) was used to view the morphological changes occurring in the fibroblast culture. Fuji Neopan 400 film was used for photography.

*Flow cytometry.* Fibroblasts for flow cytometric analysis was prepared by trypsin (0.125%) treatment followed by ethanol fixing for 20 min at 4°C and subjected to routine protocol. The cell number was determined with a Coulter Epics Elite Esp flow cytometer. 488 nm line of an argon ion laser operating at a continuous output was used for excitation.

*ELISA for 8-OH dG.* ELISA was conducted on polystyrene 96-well flat bottom plates (Nunc-ImmunoPlate Maxisorb) using the 8-OH dG ELISA kit (Japan Institute for Control of Aging, Fukuroi City, Japan). A monoclonal IgG (T45.1 clone) specific for 8-OH dG was used in this ELISA in combination with horse radish peroxidase conjugated anti-mouse polyclonal IgG and substrate *o*-phenylenediamine. The absorbance was measured at 492 nm using a computerized ELISA reader (MPR A4, Toyo Soda, Tokyo). The 8-OH dG standards used for the assay ranged between 0.64 and 2000 ng/ml. The concentration of 8-OH dG in the test samples were interpolated from the standard curve using log transformation.

*Statistical analyses.* 8-OH dG concentration in the culture medium samples obtained in triplicate experiments were expressed as mean ± SEM. Time sequential variation in the 8-OH dG within the treatments (control medium and medium enriched with 30 mM carnosine) was compared by ANOVA and post-hoc Duncan's Multiple Range test. Values between the two treatments were analyzed by Mann-Whitney's U-test. A value of $P < 0.05$ was identified as significant.

## RESULTS AND DISCUSSION

Microscopical observation on the morphological changes occurring in fibroblasts grown in DMEM without nutritional supplement is shown in Fig. 1. Whereas the cells grown in the control medium lost their characteristic morphology after one week (Fig. 1A), the fibroblasts subjected to the same degree of nutritional insult, but grown in medium supplemented with 30 mM L-carnosine, retained the healthy appearance (Fig. 1D). By three weeks, the cells grown in the control medium had lost cellular integrity (Fig. 1B) and ultimately lost their viability in four weeks (Fig. 1C). Contrastingly, cellular integrity was preserved in fibroblasts grown in medium supplemented with 30 mM L-carnosine even after four weeks (Fig. 1E and 1F). However, carnosine-treated fibroblasts enlarged in their appearance with time. The reason for this occurrence is unclear now. Those fibroblasts grown in DMEM with 10 mM L-carnosine for five weeks showed a morphology intermediate to that of controls and those grown in 30 mM L-carnosine (data not shown).

The cell viability profile, as obtained by flow cytometric measurements, of cell number among the three treatments at the end of three weeks suggested that the percentage of cell damage occurring in controls was noticeably higher in comparison to the fibroblasts grown in DMEM with 30 mM L-carnosine (Fig. 2). The cell viability profile of fibroblasts grown in 10 mM L-carnosine was intermediate to that of controls and those grown in 30 mM L-carnosine. The flow cytometric profiles also indicate the differences in the concentration of 8-OH dG remaining in the fibroblasts which underwent different carnosine enrichment treatments. The cell number in each of the treatment is depicted by the right peak in panels B, C and D of Fig. 2. A visible shift of the right peak towards the left axis, with increasing L-carnosine concentration in the medium, suggests a decrease in the amount of 8-OH dG tagged to the cells, as bound to the primary antibody of 8-OH dG.

The 8-OH dG concentration in the culture medium of fibroblasts grown in 30 mM L-carnosine and collected at weekly intervals is shown in Table 1. Whereas fibroblasts grown in the control medium showed a significant five fold increase ($P < 0.05$) in the release of 8-OH dG after five weeks, the cells grown in the medium supplemented with 30 mM L-carnosine did not show such a comparable increase in the release of 8-OH dG. Furthermore, by the end of four weeks, the 8-OH dG released by the fibroblasts in control medium was significantly higher ($P < 0.05$) to that of the

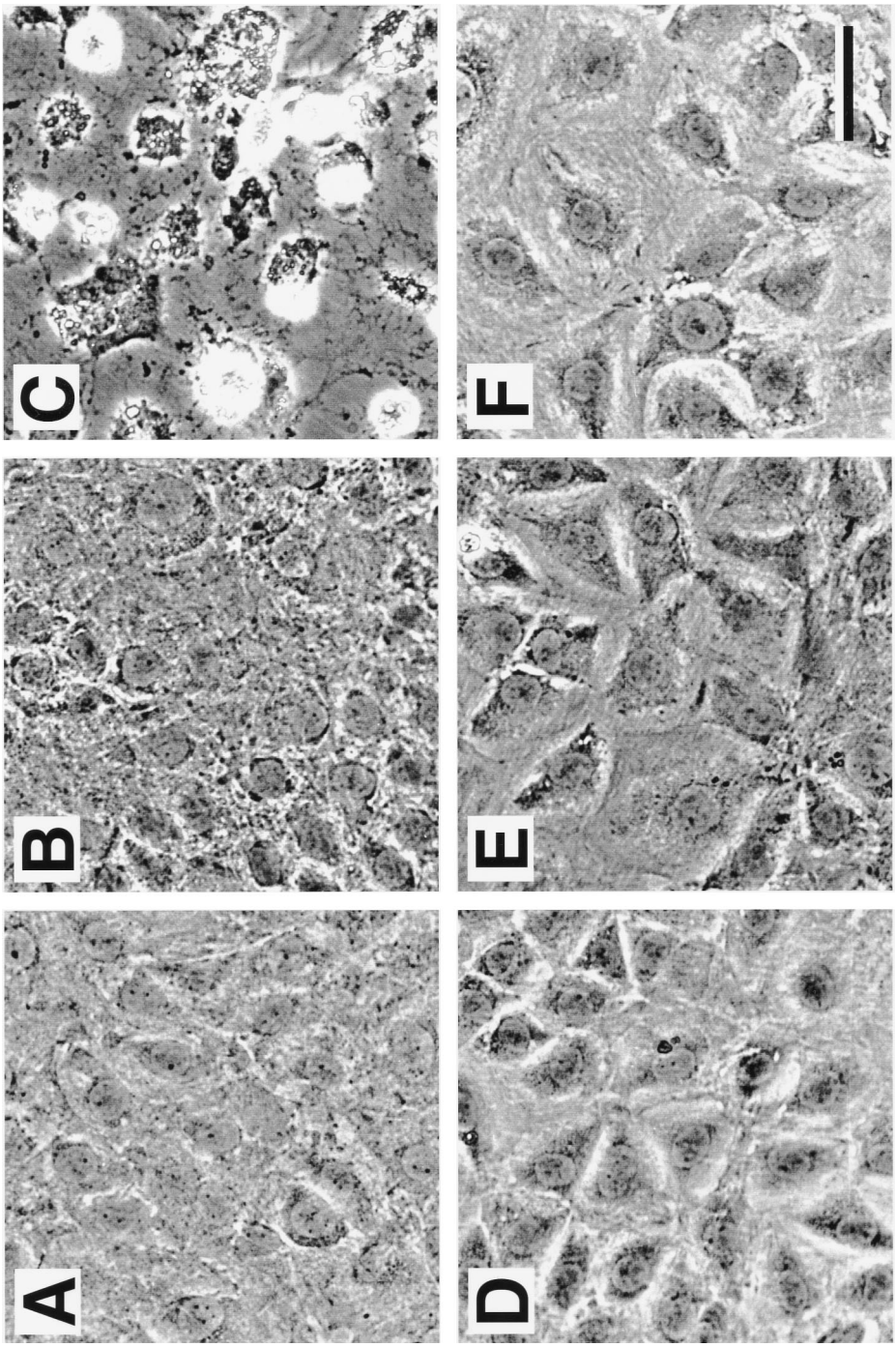


**FIG. 1.** Time course of morphological changes in fibroblasts grown in DMEM without nutritional replenishment at 37°C. Panels A-C, control medium; Panels D-F, medium supplemented with 30 mM L-carnosine. Panels A and D, 1 week; Panels B and E, 3 weeks; Panels C and F, 4 weeks. The bar represents 50 $\mu$m.

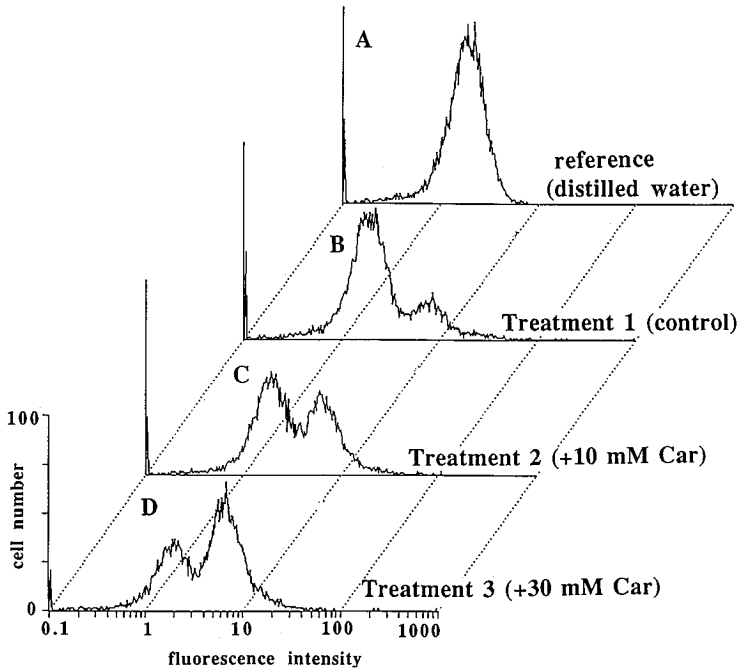


**FIG. 2.** Effect of L-carnosine on fibroblast survival after three weeks of culture in DMEM without nutritional replenishment at 37°C. Panels A-D show representative flow cytometric traces. (A) Reference signal obtained with distilled water; (B) Treatment 1, control without L-carnosine; (C) Treatment 2, with 10 mM L-carnosine; (D) Treatment 3, with 30 mM L-carnosine. These fluorescence data are expressed as histograms of events (cell number) versus fluorescence intensity. Carnosine is denoted by the abbreviation Car.

amount of 8-OH dG released by the cells grown in the medium supplemented with 30 mM L-carnosine. Previously Kohen et al. (5) had shown in an *in vitro* system that L-carnosine (1 mM) inhibited the formation of 8-OH dG from deoxyguanosine (1 mM) in the presence of $CuSO_4$ (100 $\mu$M) and ascorbic acid (1 mM). Our finding in fibroblast model corroborates the results obtained by Kohen et al.(5). Also, the microscopic evidence obtained in our study agrees with the data of McFarland and Holliday (11) that L-carnosine at 30 mM concentration has a beneficial effect in retarding the senescence of fibroblasts.

TABLE 1
8-Hydroxy Deoxyguanosine Released by the Fibroblasts with Increasing Duration of Nutritional Insult

| Duration of nutritional insult (weeks) | 8-hydroxy deoxyguanosine concentration[a] (ng/ml) | |
|---|---|---|
| | Control medium | Medium with 30 mM Car |
| 1 | $4.00 \pm 0.35^{A}$ | $4.69 \pm 0.64$ |
| 2 | $9.26 \pm 3.65^{A}$ | $6.34 \pm 0.72$ |
| 3 | $11.73 \pm 5.69$ | $5.24 \pm 0.26$ |
| 4 | $11.03 \pm 2.86^{a}$ | $5.87 \pm 0.77^{b}$ |
| 5 | $22.59 \pm 3.14^{B}$ | $13.00 \pm 4.99$ |

[a] Mean ± SEM; n = 3. Means with different superscript letters are significantly different at $P < 0.05$. Capital letters denote comparison within columns, lower-case letters denote comparison between two treatments. Absence of superscripts indicate no significant difference either within columns or between two treatments. L-Carnosine is denoted by the abbreviation Car.

In sum, based on three different experimental approaches (microscopic, flow cytometric and ELISA for one of the markers of DNA oxidative damage), our study reveals that L-carnosine could sustain the retention of cell morphology in continuous fibroblast culture subjected to nutritional insult. The probable biochemical mechanisms which are involved in the reduction of formation of 8-OH dG in fibroblast culture due to carnosine treatment are not clear at present. Studies are currently in progress to identify these mechanisms.

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