

## Carnosine Stimulates Vimentin Expression in Cultured Rat Fibroblasts

Daisuke Ikeda<sup>1</sup>, Shun-ich Wada<sup>1</sup>, Chie Yoneda<sup>1</sup>, Hiroki Abe<sup>2</sup>, and Shugo Watabe<sup>1\*</sup>

<sup>1</sup>Laboratory of Aquatic Molecular Biology and Biotechnology, <sup>2</sup>Laboratory of Marine Biochemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113–8657, Japan

**ABSTRACT.** Two-dimensional electrophoretic gel profiles were compared between rat 3Y1 fibroblasts cultured in the presence and absence of 30 mM L-carnosine ( $\beta$ -alanyl-L-histidine) for one week without any replenishment of medium. While a number of cellular proteins changed their expression levels by the addition of carnosine, we identified one of the most prominently varied proteins as vimentin. Immunoblot analysis with anti-vimentin antibody demonstrated that the vimentin levels increased about 2-fold after one-week culture in the presence of carnosine. We also confirmed that the increase of vimentin expression was dependent on the concentration of carnosine added to the medium. Moreover, when cultured cells were stained with anti-vimentin antibody and observed by light microscopy, most cells grown in the presence of carnosine were found to have markedly developed vimentin filaments. The increase of vimentin expression was also observed by adding with carnosine related dipeptides, *N*-acetylcarnosine and anserine.

**Key words:** carnosine/vimentin/cell culture/rat fibroblast

The dipeptide L-carnosine ( $\beta$ -alanyl-L-histidine) was first isolated from meat extract in 1900 and is known to be contained in skeletal muscle tissues of vertebrates at particularly high concentrations ( $\sim 20$  mM) (1–3). While the carnosine related dipeptides, anserine ( $\beta$ -alanyl- $\pi$ -methyl-L-histidine) and balenine ( $\beta$ -alanyl- $\tau$ -methyl-L-histidine), are also distributed in skeletal muscle, *N*-acetylated derivatives of carnosine, *N*-acetylcarnosine, is specifically found in myocardial tissues (1–4). Carnosine has been claimed to have various physiological functions: e.g., it functions as a proton buffer (3), activates myosin ATPases (5), participates as a neurotransmitter (6), and has antioxidant activity (7–12). However, the exact physiological roles of carnosine and related dipeptides in living cells still remain ambiguous.

McFarland and Holliday (13) reported that adding

carnosine to the medium retarded the senescence of cultured human fibroblasts and rejuvenated the cells. In a recent study, they observed that carnosine inhibited the growth of transformed and neoplastic cells *in vitro* along with the reduction in concentrations of glycolysis intermediates (14). As the metabolism of tumor cells is much more dependent on anaerobic glycolysis than that of normal cells, such changes for glycolysis intermediates in the presence of carnosine seem to be highly cytotoxic for transformed and neoplastic cells (14).

In our previous study, rat fibroblasts of the 3Y1 cell line retained cell morphology even during long-term culture for 4 weeks under nutritional insult in the presence of carnosine (15). Such antisenescence effects of carnosine were proposed to be linked to the reduction of the oxidative damage for DNA where one of the oxidative products, 8-hydroxy deoxyguanosine, was traced (15).

While a few studies have been performed on the effects of carnosine on cultured cells, they only focused on cell morphology, growth, or uptake and synthesis of carnosine (13–16). In this study, we employed two-dimensional electrophoretic analysis to disclose the effects of carnosine on cultured rat 3Y1 fibroblasts in the medium supplemented and not supplemented with carnosine. Several proteins were varied when cells were treated with 30 mM carnosine for one week, and one

\* To whom correspondence should be addressed: Laboratory of Aquatic Molecular Biology and Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan.

Tel: +81–3–3812–2111 ext. 7520, Fax: +81–3–5684–0622

E-mail: awatabe@hongo.ecc.u-tokyo.ac.jp

Abbreviations: DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TFA, trifluoroacetic acid.

of the increased proteins was identified as vimentin, a component of intermediate filaments.

## Materials and Methods

### Materials

Unless otherwise stated, all chemicals used in this study were purchased from Wako Pure Chemicals (Tokyo). Anserine was purified by the previously reported method (17) and *N*-acetylcarnosine was kindly provided from Dr. A.A. Boldyrev (Moscow State University).

### Cell culture

Rat embryonic fibroblasts of the 3Y1-B clone 1–6 (18) or Chinese hamster ovarian fibroblasts of CHO-K1 cell line (19) were plated onto culture dishes at a density of  $1.35 \times 10^7$  cells/150-mm plate or  $5 \times 10^5$  cells/100-mm plate. 3Y1 fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM) (GIBCO, Gaithersburg, MD, USA) and CHO fibroblasts in DMEM/F-12 (GIBCO) supplemented with 10% fetal calf serum (Bio Whittaker, Walkersville, MD, USA), penicillin (100 U/ml), streptomycin (100 µg/ml), amphotericin B (0.25 µg/ml) and gentamicin (75 µg/ml) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C.

Cultured cells were supplemented with various concentrations of carnosine and related compounds in the DMEM and allowed to grow further for certain periods without nutritional replenishment in order to examine possible effects of carnosine on the cells.

### Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed under the conditions described by O'Farrell (20), using 4% polyacrylamide gels in the presence of 9 M urea with 5.3% Ampholine distributed from pH 3.5 to 10.0 (Pharmacia, Uppsala, Sweden) for the first dimensional isoelectric focusing. The second dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 12.5% slab gels. Gels were stained with 0.1% Coomassie Brilliant Blue R250 after electrophoresis. The total protein of about 300 µg was loaded on each isoelectric focusing gel.

### Immobilization of proteins onto polyvinylidene difluoride (PVDF) membranes

Approximately 2 × 2 mm protein spots of interest were excised from fifteen Coomassie-stained gels, and the proteins were concentrated on a gel system described by Vandekerckhove and Rasmussen (21). Concentrated proteins were electrically transferred onto Immobilon PVDF membranes (Millipore, Bedford, MA, USA) and stained with 0.1% Ponceau S dye in 1% aqueous acetic acid.

### *In situ* digestion of PVDF-immobilized protein

The method described by Fernandez *et al.* (22) was employed. Protein-containing bands were cut out with a clean razor, transferred to microtubes (1.5 ml), and destained by washing the membranes with 500 µM NaOH in 20% acetonitrile for 1 min, followed by an additional wash with distilled water. The membranes were incubated with 500 µl of 0.2% polyvinylpyrrolidone (Mr 40,000) in methanol (w/v) at room temperature for 30 min, followed by the addition of 500 µl distilled water. After incubation at 37°C for 30 min, the membranes were washed 10 times each with 1 ml distilled water and cut into approximately 1 mm squares. To the strips were added 50 µl 100 mM Tris-HCl (pH 8.0) containing 1% hydrogenated Triton X-100 (Sigma, St Louis, MO, USA), 10% acetonitrile, and 5 µl of trypsin at a final concentration of 0.1 µg of enzyme/estimated µg of substrate. Digestion was carried out at 37°C for 24 h. The digests were sonicated for 5 min and centrifuged at 200 × g for 5 min, and the supernatant was transferred to a fresh microtube. Consecutive washings with 50 µl of digestion buffer (once) and 50 µl of 0.05% trifluoroacetic acid (TFA) (twice) were performed under sonication, followed by centrifugation as described above. All supernatants were pooled for a total of 200 µl.

### Peptide mapping by reverse-phase HPLC and determination for N-terminal amino acid sequence

Peptide mapping was performed by the method of Iwamatsu and Yoshida-Kubomura (23). Peptides produced by *in situ* trypsin digestion were subjected to reverse-phase HPLC using a Wakosil-II AR (C18 300 Å, 2.0 × 100 mm) column with a Tosoh PK-8010 liquid chromatograph. Peptides were eluted at a flow rate of 0.2 ml/min with a linear gradient for 120 min using solvent A containing 0.05% TFA in water and solvent B containing 0.02% TFA in 2-propanol: acetonitrile (7:3, v/v). The eluted peptides were collected manually by monitoring absorbance at 215 nm and subjected to an Applied Biosystems model 477 A protein sequencer with an on-line system model 120 A.

### Immunoblotting

The mouse monoclonal antibody against rat vimentin (4H4) was kindly provided from Dr. M. Inagaki (Aichi Cancer Center Research Institute). Cells were harvested and lysed with a lysis buffer containing 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl (pH 7.4), and protease inhibitors of 1 µg/ml leupeptin, 1 µg/ml chymostatin, 1 µg/ml E-64, 1 µg/ml pepstatin A and 0.2 mM phenylmethylsulfonyl fluoride. Aliquots of cell lysates were subjected to SDS-PAGE (24), and electroblotted onto a PVDF membrane (25). The membrane was incubated overnight at room temperature in 10 mM Tris-HCl (pH 7.4) containing 0.9% NaCl (TBS) in the presence of 0.1% Tween 20 and 2% nonfat dry milk, and subsequently treated with monoclonal antibody 4H4 at 200-

fold dilution in TBS containing 0.1% Tween 20 (TBS-Tween) for 2 h at room temperature. After washing with TBS-Tween, the membrane was incubated with rabbit anti-mouse IgG peroxidase conjugate for 1 h at room temperature, washed with TBS-Tween, and reacted with ECL<sup>TM</sup> Western blotting detection reagents (Amersham).

Student's *t*-test was employed to compare differences in data for immunoblot analysis.

### Visualization of vimentin filaments

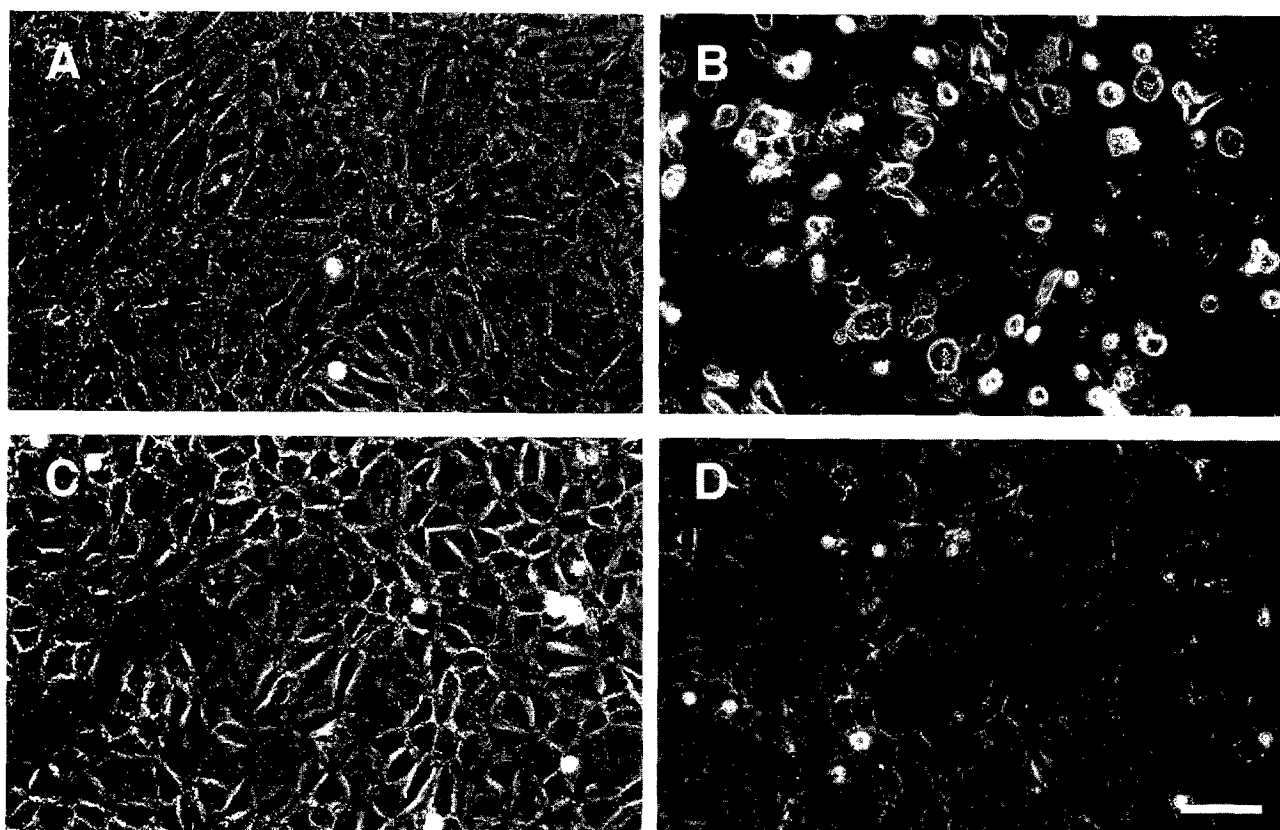
For visualization of vimentin filaments, cells were cultured on glass coverslips (15 mm in diameter), fixed in methanol for 20 min at  $-20^{\circ}\text{C}$ , and permeabilized with phosphate-buffered saline (PBS) containing 0.1% Triton X-100 for 5 min on ice. After washing twice with PBS, cells were treated with 5% bovine serum albumin for 1 h at room temperature to avoid nonspecific reaction. The cells mounted on coverslips were added with the mouse monoclonal antibody against rat vimentin diluted 4-fold in PBS, and incubated for 90 min at room temperature. After washing three times with TBS-Tween, vimentin filaments were visualized with fluorescein isothiocyanate-conjugated secondary antibodies reactive with

mouse IgG (Sigma). The fluorescence-stained coverslips were immersed into glycerol/PBS (7:3, v/v) and observed with a Nikon Labophoto-2 equipped with a Nikon HB-10101 AF fluorescence apparatus.

## Results

### Cellular Morphology

The morphological differences between rat 3Y1 fibroblasts cultured in DMEM supplemented and not supplemented with 30 mM carnosine were apparent after one week as observed in our previous paper (Fig. 1A and B) (15). Cells in the medium supplemented with 30 mM carnosine were elongated and enlarged in shape, compared to those cultured in the control medium. After four weeks, carnosine-treated cells were still alive and adhered onto culture dishes (Fig. 1D), while control cells had lost their viability. These differences were also observed with CHO fibroblasts cultured in DMEM/F-12 supplemented and not supplemented with 30 mM carnosine (data not shown).



**Fig. 1.** Morphological changes of rat 3Y1 fibroblasts cultured for one (A, C) or four weeks (B, D) in the medium not supplemented (A, B) or supplemented (C, D) with 30 mM carnosine. The bar represents 50  $\mu\text{m}$ .

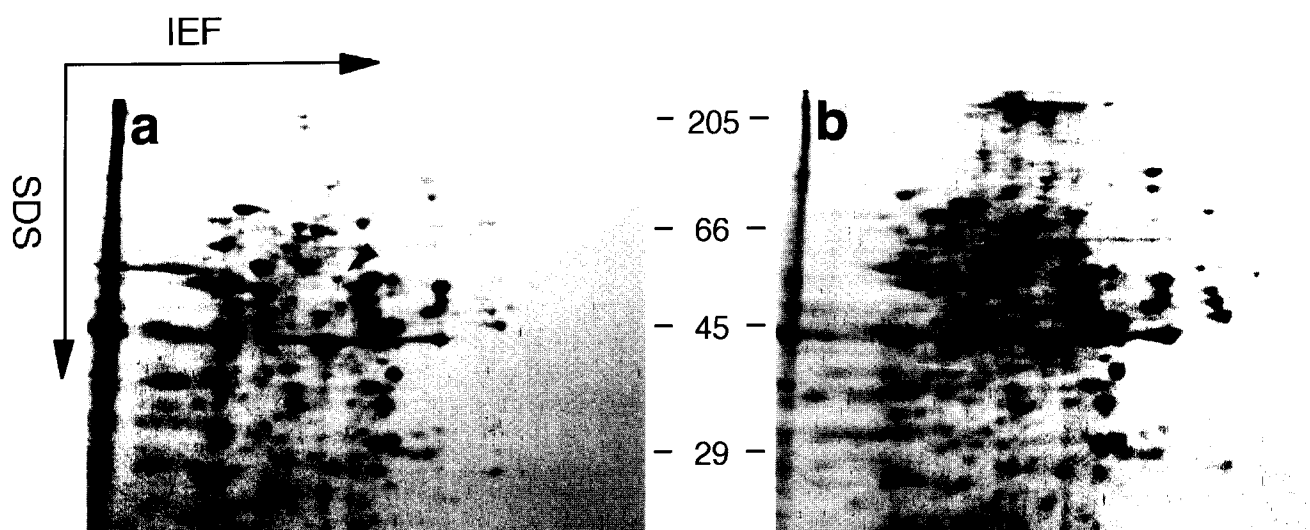


Fig. 2. Two-dimensional electrophoretic gel profiles of proteins from rat fibroblasts cultured for one week in the medium not supplemented (a) and supplemented with 30 mM carnosine (b). An arrowhead indicates a protein spot dominating in the cells cultured in the carnosine-containing medium.

### Two-dimensional Electrophoresis and N-terminal Amino Acid Sequencing

Two-dimensional electrophoresis was employed to analyze the effects of carnosine on the cellular protein composition of fibroblasts. A comparison of the gel profiles in 3Y1 fibroblasts cultured in the medium not supplemented and supplemented with 30 mM carnosine revealed differences in protein expression between the two conditions adopted (Fig. 2).

We tried to identify a 55 kDa protein, one of proteins that showed increased expression levels with the carnosine treatment (Fig. 2b, arrowhead). To obtain a sufficient amount of the protein for identification, we

collected the protein spots from fifteen gels, whereas the protein concentrated using a concentration gel system was immobilized onto PVDF membranes by semi-dry electroblotting (21). Digestion with trypsin produced several peptide fragments which could be isolated by HPLC. Three of these were applied to N-terminal amino acid sequencing analysis, giving sequences of SYVTTSTR, QQYESVAAK, and GTNESLER (Fig. 3, underlined). Homology search with the Swiss-Prot sequence database resulted in identification of the 55 kDa protein as rat vimentin (26). The occurrence of lysine or arginine as a neighboring residue to the N-terminus of the peptide further confirmed our identification, since trypsin hydrolyses ei-

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STRSVSSSSY RRMFGGSGTS SRPSSNRSYV TTSTRTYSLG SALRPSTSRs LYSSSPGGAY 60
VTRSSAVRLR SSMPGVRLlQ DSVDFSLADA INTEFKNTRT NEKVELQELN DRFANYIDKV 120
RFLEQQNKIL LAELEQLKGQ GKSRLGDLYE EEMRELRRQV DQLTNDKARV EVERDNLAED 180
IMRLREKLQE EMLQREEAES TLQSFRQDVD NASLARLDLE RKVESLQEEI AFLKKLHDEE 240
IQELQAQIQE QHVQIDVDVS KPDLTAALRD VROOYESVAA KNLQEAEEWY KSKFADLSEA 300
ANRNNDALRQ AKQESNEYRR QVQSLTCEVD ALKGTNESLE RQMREMEENF ALEAANYQDT 360
IGRLQDEIQN MKEEMARHLR EYQDLLNVKM ALDIEIATYR KLEGEESRI SLPLPNFSSL 420
NLRETNLES� PLVDTHSKRT LLIKTVETRD GQVINETSQH HDDLE 465

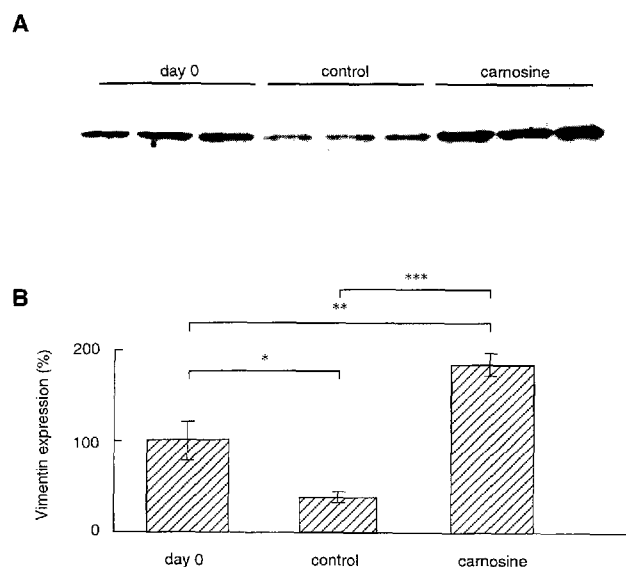
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Fig. 3. Identification of the 55 kDa protein with increased levels following carnosine treatments of rat fibroblasts by N-terminal amino acid sequencing for tryptic peptides. Amino acid sequence of the rat vimentin fragments were retrieved from the Swiss-Prot sequence database. The amino acid sequences determined in this study are underlined. Note that the C-terminal neighboring residue of each fragment is lysine (K) or arginine (R).

ther lysyl or arginyl bond. The apparent molecular weight of this protein as revealed by electrophoresis was consistent with that reported for rat vimentin (26).

### Immunoblot Analysis of Vimentin

Immunoblotting was employed to determine the expression levels of vimentin in rat 3Y1 fibroblasts cultured in the medium containing carnosine. The monoclonal antibody against rat vimentin 4H4 reacted specifically with vimentin and no other protein bands were detected in our system (Fig. 4A). The vimentin levels increased about 2-fold ( $P < 0.01$ ) after one-week culture in the presence of 30 mM carnosine, while they reduced to about 1/3 ( $P < 0.005$ ) of the initial level in the absence of carnosine (Fig. 4B). Total proteins per plate were about 1.9-fold higher in cells not supplemented with carnosine than carnosine-treated cells after one-week culture, whereas cell numbers were about 1.5-fold higher than those of the latter. Therefore, protein contents per cell were about 1.3-fold higher in cells cultured without carnosine than those with carnosine. The same protein quantities of 7.5  $\mu$ g were applied to immunoblot analysis for both the above two experimental groups. Thus, vimentin expression levels per cell as revealed by immunoblotting were still significantly



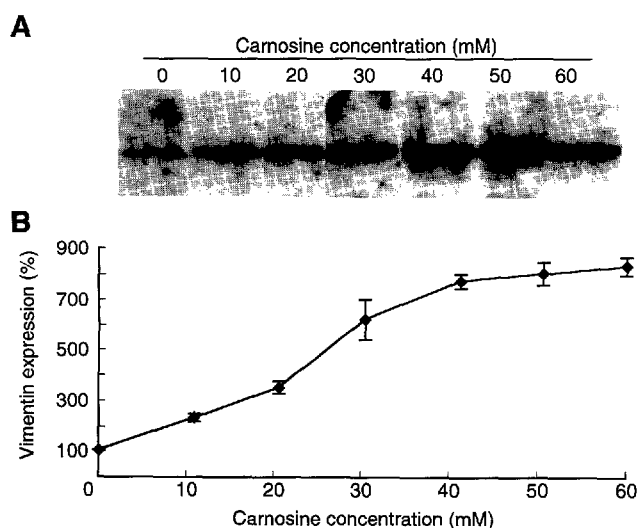
**Fig. 4.** Immunoblot analysis of vimentin in rat fibroblasts supplemented with carnosine. Total proteins were prepared from cells at the start of culture (day 0) and those cultured for one week in the medium supplemented (carnosine) and not supplemented (control) with 30 mM carnosine. SDS-PAGE was carried out with 7.5  $\mu$ g proteins for each lane followed by immunoblotting using the antibody raised against rat vimentin (A). Data in B are means  $\pm$  standard errors ( $n=3$ ) for relative vimentin levels with significant differences at  $P < 0.01$  (\*),  $P < 0.005$  (\*\*), and  $P < 0.0001$  (\*\*\*).

higher in carnosine-treated cells than those without treatment. Such significant changes of vimentin expression levels were further confirmed when vimentin concentrations were compared with those of  $\beta$ -actin (data not shown).

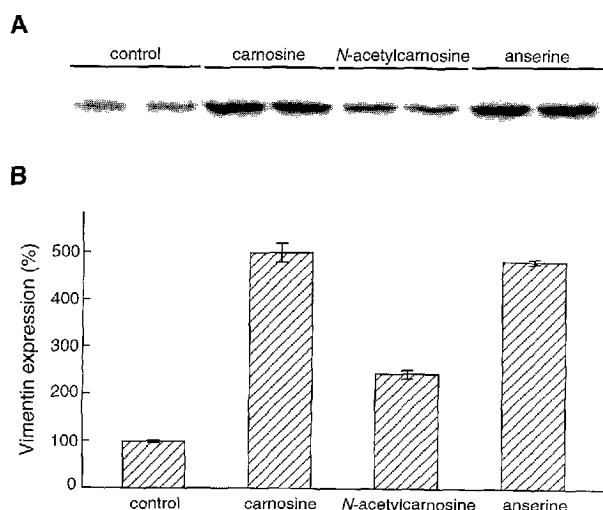
We tried to observe the carnosine concentration dependency of vimentin expression in 3Y1 fibroblasts. Proteins were extracted from fibroblasts cultured for one week in the medium supplemented with 0–60 mM carnosine, and 7.5  $\mu$ g was subjected to immunoblotting (Fig. 5). The expression levels of vimentin were increased in a carnosine concentration-dependent manner and reached almost the maximum at 40 mM (Fig. 5B). We also examined the effect of two carnosine-related dipeptides, *N*-acetylcarnosine and anserine, on vimentin expression (Fig. 6). Although the increase of vimentin expression was observed for the two dipeptides, *N*-acetylcarnosine was less effective than carnosine and anserine (Fig. 6B).

### Cellular Organization of Vimentin

Organization of the vimentin filaments in rat 3Y1 fibroblasts was observed by immunofluorescence microscopy (Fig. 7). The addition of 30 mM carnosine into the medium resulted in the development of vimentin filaments in cultured cells (Fig. 7B, arrowheads). In the control medium, there were few cells showing such vimentin organization in the observed microscopic fields. In addition, cells cultured in the medium containing



**Fig. 5.** Carnosine concentration-dependency of vimentin expression in rat fibroblasts. Total proteins were prepared from fibroblasts cultured for one week in the medium supplemented with 0, 10, 20, 30, 40, 50 and 60 mM carnosine, and subjected to SDS-PAGE followed by immunoblotting (7.5  $\mu$ g proteins per lane) (A). Data in B are means  $\pm$  standard errors ( $n=4$ ) for relative vimentin levels.



**Fig. 6.** Immunoblot analysis of vimentin in rat fibroblasts supplemented with carnosine and related dipeptides. Total proteins were prepared from cells cultured for one week in the medium not supplemented (control) and supplemented with 30 mM carnosine, *N*-acetylcarnosine, or anserine. SDS-PAGE was carried out with 7.5  $\mu$ g proteins per lane followed by immunoblotting using the antibody raised against rat vimentin (A). Data in B are means  $\pm$  standard errors ( $n=4$ ) for relative vimentin levels.

carnosine were more elongated and flattened than those in the control medium.

## Discussion

Our purpose in this study was to reveal the effects of carnosine on protein expression in rat 3Y1 fibroblasts *in vitro* in order to understand its possible physiological functions *in vivo*. We observed such effects by two-dimensional electrophoretic analysis, and found that various cellular proteins changed their levels in a carnosine-dependent manner. One of these proteins was vimentin, the increased levels of which were confirmed by immunoblotting using a specific monoclonal antibody. These changes were also observed with the two carnosine-related dipeptides, *N*-acetylcarnosine and anserine. In addition, immunofluorescence microscopy showed that most cells extensively developed vimentin filaments in their cytoplasm when a medium was supplemented with carnosine.

Vimentin belongs to type III intermediate filaments and is thought to play a fundamental role in maintaining cell structure and integrity (27). In cell cycle, cdc2 kinase phosphorylates vimentin filaments and induces the disassembly of these filament structures at mitotic phase (28–30). Ben-Ze'ev (31) reported that in various cell lines including B16 melanoma, 3T3 fibroblasts, BSC-1 kidney cells, and SV 40-transformed 3T3, biosynthesis of vimentin was selectively reduced during

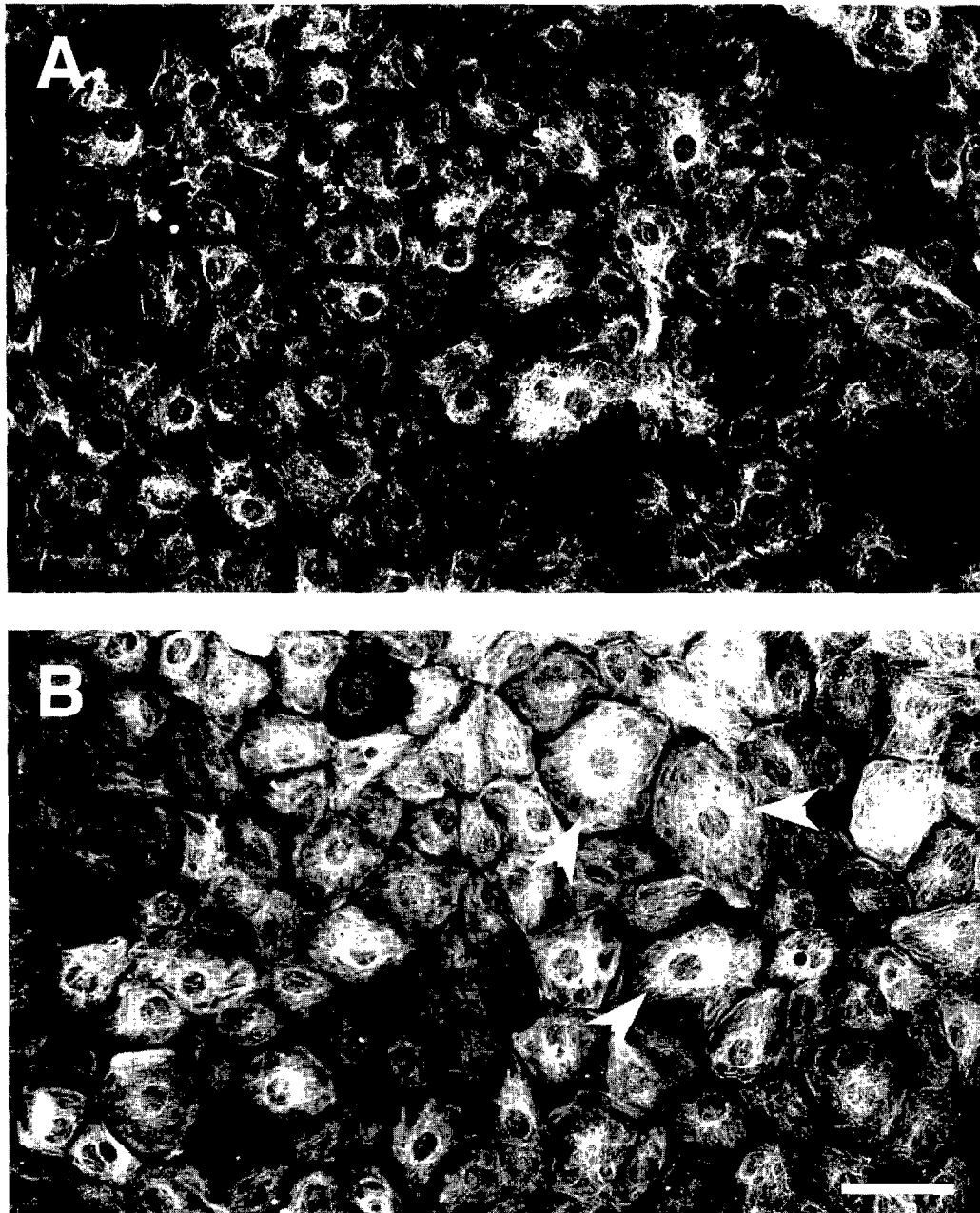
suspension culture, but was rapidly increased upon reattachment of cells to the substrate. It was also claimed that in the dense cell culture, vimentin expression was highly reduced compared to sparse conditions (32). It has been known that one of the most remarkable morphological changes of *in vitro* senescent cells was the increase of cell size (33–35), and that the senescent cells also increased the expression of vimentin at either mRNA or protein levels (36, 37). These phenomena suggest that the attached cell areas as well as adhesion are strongly related to the expression of vimentin.

The vimentin gene was isolated from rat as the one highly expressed in aggressive prostatic tumors, but poorly in normal ones (26). Hendrix *et al.* (38) showed that experimental overexpression of vimentin led to invasive and metastatic behavior of MCF-7 human breast cancer cells which were not originally endowed with such properties. However, the relationship between cancer and vimentin expression has not been clearly elucidated. For example, vimentin synthesis was reduced in murine erythroleukemia cells, while it was induced in chicken erythroleukemia and human promonocytic leukemia during differentiation (39, 40). Colucci-Guyon *et al.* (41) produced mice lacking the vimentin gene to address the biological roles of vimentin. Surprisingly, such mice having no corresponding intermediate filaments were able to develop and reproduce without any apparent changes in phenotypes.

The attached areas of rat 3Y1 fibroblasts which were cultured in the carnosine-containing medium and developed vimentin filaments, were larger than those in a control medium (see Fig. 7) (15). Cultured human fibroblasts grown in carnosine had a flat, spread-out appearance with very uniform spacing in comparison with cells not supplemented with carnosine (13). Such morphological changes were also observed with 3Y1 fibroblasts cultured in the serum-free medium (data not shown). It was also reported that the size of human diploid fibroblast-like cells from skin tissue was increased by growth reduction under low concentration of serum (0.1–1.0%) (34).

As to why the attached areas of cells cultured in the medium supplemented with carnosine were larger than those in the medium without carnosine, it has recently been indicated that reactive oxygen species (ROS) act as intracellular messengers in receptor-associated signaling pathways (42–47). It has also been shown that cellular responses mediated by ROS are inhibited by scavenging enzymes and chemical antioxidants (42–47). Carnosine also has an antioxidant activity (7–12), while growth rate of the cells treated with carnosine is reduced (13).

It has been also reported that carnosine inhibited the growth of transformed and neoplastic cells *in vitro* along with the reduction in concentrations of glycoly-



**Fig. 7.** Cellular organizations of vimentin filaments in rat fibroblasts cultured in the medium not supplemented (A) and supplemented (B) with 30 mM carnosine for one week. Note the cells indicated with arrowheads, where vimentin filaments developed extensively in their cytoplasm. The bar represents 50  $\mu$ m.

sis intermediates (14). In our case, it can also be assumed that the growth of cells was inhibited as a result of the disturbance of the cellular metabolism by carnosine.

The increases of vimentin expression were observed in cells supplemented with not only carnosine but also *N*-acetylcarnosine and anserine. The vimentin expression would be used as a good index that reflects the

effect of the three imidazole dipeptides at the molecular level. While the increased vimentin levels may result in unknown effects on cellular events following the addition of carnosine, it is important to determine the mechanisms responsible for the changes in vimentin expression for a better understanding of the physiological roles of carnosine.

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