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SELECTIVE INHIBITION OF COLLAGEN ACCUMULATION BY *N*-(3,4-DIMETHOXYCINNAMOYL)ANTHRANILIC ACID (N-5′) IN GRANULATION TISSUE

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Abstract—The effect of topically applied N-5′, an inhibitor of chemical mediator release from mast cells, on the carrageenin-air-pouch inflammation was studied. The formation of granulation tissue, the accumulation of exudate and the number of infiltrating cells were significantly reduced by the treatment with N-5′ (100 mg/kg).

The collagen content in granulation tissue was dose-dependently reduced without affecting the noncollagen protein and DNA content by treatment with N-5′. At a dose of 100 mg/kg of N-5′, prolyl hydroxylase activity in the tissue was significantly decreased. The selective inhibition of collagen accumulation in granulation tissue resulted from reduction of collagen biosynthesis *in vivo*.

N-5′ did not directly inhibit collagen synthesis by diploid fibroblasts, but inhibited fibroblast proliferation in culture. Such results indicate that one of the inhibitory mechanisms of collagen accumulation by N-5′ in inflamed sites may involve the inhibition of fibroblast proliferation.

Accumulation of collagen fibers is one of pathologic features in various chronic inflammations involving proliferative reactions. As a result of excess collagen accumulation, tissues or organs exhibit fibrosis and this makes it difficult to cure diseases such as liver cirrhosis, pulmonary fibrosis, rheumatoid arthritis, hypertrophic scars and keloid [1].

In spite of recent advances in the biochemistry of collagen synthesis [2] and degradation [3], regulation mechanisms of collagen accumulation in chronic inflammation have not been elucidated in any detail *in vivo*.

It has been reported that fibroblast proliferation and/or collagen synthesis are regulated by chemical mediators released from various inflammatory cells such as mast cells [4, 5], mononuclear cells [6, 7], lymphocytes [8, 9] and platelets [10] *in vitro* studies, and the hypothesis has been proposed that collagen accumulation in inflamed sites is modulated by the transmission of information via chemical mediators between inflammatory cells and fibroblasts [11].

Based on this hypothesis, it might be possible to inhibit collagen accumulation in inflamed sites by inhibiting chemical mediator release from inflammatory cells and/or the intracellular transmission of chemical messages in fibroblasts.

N-(3,4-dimethoxycinnamoyl)anthranilic acid (N-5′) has been used clinically as an antiasthma drug. Its pharmacological properties are the inhibition of passive cutaneous anaphylaxis *in vivo* and chemical mediator release from mast cells *in vitro* [12]. It has been noted that the inhibitory mechanism of chemical mediator release is the inhibition of the energy-requiring system and/or Ca^{2+} influx at the time of mast cell degranulation [13].

In present paper, we have attampted to evaluate the effect of N-5′ on collagen accumulation in carrageenin-air-pouch inflammation and on diploid fibroblast proliferation and collagen synthesis in culture.

MATERIALS AND METHODS

Treatment with N-5′ in vivo. Male Donryu rats, weighing 170–190 g, were used in studies *in vivo*. Carrageenin-air-pouch inflammation was induced by subcutaneous injection of 4 ml of 2% (w/v) carrageeinin (Seakem 202; Marine Colloid Inc., Springfield, NJ) solution into a preformed air-pouch on the dorsum of rats [14]. The effects of N-5′ (Kissei Pharmaceutical Co. Ltd.) on carrageeinin-air-pouch inflammation were studied by the following two procedures.

In the first procedure, 10 mg/kg/ml, 30 mg/kg/ml and 100 mg/kg/ml of N-5′ dissolved in dimethylsulfoxide (DMSO: Wako Pure Chemical Industries Ltd) were injected into the air-pouch immediately after injection of carrageenin solution (day 0) and repeatedly injected every 12 hr until day 3 (for 4 days). Control rats were injected with 1 ml/kg of DMSO. On day 4 after carrageenin injection, the animals were sacrificed, the volume of pouch fluid, the wet weight of granulation tissue and the number of infiltrating cells (using Toa microcell counter CC-120) were determined.

In the second procedure, 100 mg/kg/ml of N-5′ was injected every 12 hr from day 0 to day 3 (Group 1), from day 0 to day 1 (Group 2) and from day 2 to day 3 (Group 3). On day 4, each animal was sacrificed, and the effects of N-5′ on the various phases of carrageenin-air-pouch inflammation were observed.

The collagen in granulation tissue was extracted twice as a gelatin by autoclaving the tissue at 110° for 1 hr, and the residue of the tissue was referred to as noncollagen protein [15]. The content of hydroxyproline in the collagen fraction was determined by the method of Kivirikko *et al.* [16] and

the content of noncollagen protein was measured according to Lowry's method [17]. The content of DNA in the homogenated granulation tissue was determined according a modification of Burton's method [18]. A portion of the granulation tissue was fixed by 10% buffered formalin. Specimens were embedded in paraffin, sectioned and stained with Azan-mallory stain.

Assay of prolyl hydroxylase activity. An aliquot of minced granulation tissue was homogenized in 10 vol. of 20 mM Tris–HCl (pH 7.5) containing 0.2 M NaCl and 0.1 M glycine with an ultradisperser (Yamato Co. Ltd.). Two volumes of the homogenate buffer containing 0.15% Triton X-100 were added to the homogenate solution which was let stand at 0° for 30 min and centrifuged at 15,000 *g* for 30 min. Prolyl hydroxylase activity in the supernatant was measured by tritiated water release according to the method of Hutton *et al.* [19]. The assay system consisted of 20 μl of the supernatant (about 30 μg of protein), 40 mM Tris–HCl (pH 7.6), 1.0 mM sodium ascorbate, 1 mM α-ketoglutalate, 0.2 mM ferrous ammonium sulfate, 0.4 mg/ml of catalase, 2.0 mg/ml of bovine serum albumin, 0.5 mM dithiothreitol and 10^5 dpm of L-(3,4-^{3}H)proline-labeled protocollagen substrate at a final volume of 1 ml. Incubation was carried out at 30° for 30 min, the tritiated water released from the reaction mixture was collected by vacuum distillation and the radioactivity was measured by a liquid scintillation spectrometer.

Diploid fibroblast culture. Diploid fibroblasts were obtained from human embryonic skin and subcultured in Dulbecco's modified Eagle's medium (DMEM: Flow Lab. Inc., U.S.A.) supplemented with 10% fetal bovine serum (FBS: Flow Lab. Inc., U.S.A.) in a 95% humidified air and 5% CO_2 atmosphere at 37°.

Effect of N-5′ *on fibroblast proliferation.* A plastic plate with 4.3×10^4 fibroblasts/25 cm^2 (four passages) in 2.5 ml of DMEM containing 5% FBS was used for the culture. After incubation for 2 days, the medium was aspirated, and 2.5 ml of fresh medium and 0.1 ml of N-5′ solution were added to the culture plate. N-5′ was dissolved in 1% (w/v) $NaHCO_3$ solution, and diluted with DMEM containing 5% FBS to final concentrations of 1 μg/ml, 10 μg/ml and 100 μg/ml.

On days 4 and 6, fibroblasts were trypsinized with 0.2% trypsin (Difco Lab.) in phosphate buffered saline [PBS(−): Nissui Seiyaku Co. Ltd.] and the cells were counted using a hemocytometer.

Effect of N-5′ *on fibroblast collagen and noncollagen protein synthesis.* Fibroblasts grown to confluency (four passages: 3.6×10^5 cells/25 cm^2 of plastic plate) were incubated with DMEM containing 5% FBS and 50 μg/ml of sodium ascorbate. After 1 hr of incubation, the medium was replaced with 2.5 ml of fresh medium containing in addition 100 μg/ml of β-aminopropionitrile (Nakarai Co. Ltd.) and 1 μCi/ml of L-(5-^{3}H)proline (Amersham, 31 Ci/mmol). The incubation was continued for 3, 6 and 9 hr.

Effects of N-5′ (final concentrations: 1, 10 and 100 μg/ml) on the collagen and noncollagen protein synthesis were studied during 6 hr of incubation.

At the end of the incubation, the contents of radioactive collagen and noncollagen protein in the medium and the cell layer were determined by the bacterial collagenase digestive method according to the procedure of Peterkofsky and Diegelmann [20]. The cell layer was exposed to 0.5 N NaOH solution for neutralization, protease-free ribonuclease (Sigma, Type XII-A) was added to give a final concentration of 10 μg/ml for cleaving aminoacyl-tRNA and the solution was incubated at 37° for 5 min. Then 0.05 ml of FBS was added as a carrier protein. An equal volume of 10% trichloroacetic acid (TCA) containing 0.5% tannic acid was added each medium and cell layer treated with ribonuclease. The supernatant was removed by centrifugation, and washed with 3 ml of 5% TCA–0.25% tannic acid, 5% TCA and twice with ethanol/ether 3:1 (v/v). The dried precipitate was dissolved in 3 ml of 0.1 N NaOH solution and vortexed.

The reaction mixture consisted of 0.2 ml of the sample solution, 0.05 ml of 1.2 M Hepes buffer (pH 7.2) containing 25 mM *N*-ethylmaleimide (Nakarai Co. Ltd.) and 50 mM $CaCl_2$, 0.2 ml of 0.08 N HCl and 0.05 ml (18 μg of protein) of collagenase (Sigma, Type VII) purified by Sephadex G-200 in 0.05 M Tris–HCl (pH 7.6) containing 5 mM $CaCl_2$. In the case of the blank, 0.05 ml of 0.05 M Tris–HCl (pH 7.6) containing 5 mM $CaCl_2$ was added in place of the collagenase solution. Collagenase-sensitive protein and collagenase-insensitive protein were referred to as collagen and noncollagen protein respectively.

RESULTS

Effect of N-5′ *on carrageenin-air-pouch inflammation*

Doses of 10 mg/kg, 30 mg/kg and 100 mg/kg of N-5′ dissolved in DMSO were injected into the preformed air-pouch immediately after injection of carrageenin solution and repeatedly injected every 12 hr for 4 days. The results are summarized in Table 1.

The accumulation of pouch fluid, formation of granulation tissue and number of cells in the pouch fluid were significantly reduced by treatment with 100 mg/kg of N-5′ when compared with the control group repeatedly injected 1 ml/kg of DMSO.

The contents of collagen, noncollagen protein and DNA in granulation tissue are shown in Table 2. The content of hydroxyproline in the whole granulation tissue was dose-dependently reduced by N-5′ treatment, and significant inhibition of collagen accumulation by 43% was observed at a dose of 100 mg/kg of N-5′. Such a tendency was also seen in the determination of hydroxyproline content per gram of tissue and significant inhibition of 27% was observed in the same group.

On the other hand, both contents of noncollagen protein and DNA in the whole granulation tissue were not affected by treatment with N-5′, and those per gram of tissue showed a slight tendency to increase dose-dependently without any significant difference.

The collagen–noncollagen protein ratio was dose-dependently decreased, and a significant decrease was observed at doses of 30 mg/kg and 100 mg/kg of N-5′ (Table 2).

Table 1. Effect of N-5′ on carrageenin-induced inflammation in rats

	Body wt (g)	Pouch fluid (g)	Granulation tissue, wet wt (g)	Total cells ($\times 10^8$)
Control (DMSO)	216 ± 4	13.9 ± 1.0	3.95 ± 0.32	4.08 ± 0.27
N-5′				
10 mg/kg	221 ± 2	13.9 ± 0.9	3.67 ± 0.12	4.46 ± 0.57
30 mg/kg	220 ± 3	13.5 ± 1.5	3.75 ± 0.20	3.21 ± 0.31
100 mg/kg	217 ± 3	9.9 ± 0.5†	3.08 ± 0.22*	2.29 ± 0.28†

N-5′ dissolved in dimethylsulfoxide (DMSO) was injected into the carrageenin-air-pouch immediately after carrageenin injection and the injection was repeated every 12 hr until day 3 (8 injections in total). Data are shown as means ± SE. Values are significantly different from the control: * $P < 0.05$, † $P < 0.01$.

Table 2. Effect of N-5′ on collagen accumulation and prolyl hydroxylase activity in granulation tissue

	Control (DMSO)	N-5′ (mg/kg)		
		10	30	100
Granulation tissue wet weight (g)	3.95 ± 0.32	3.67 ± 0.12	3.75 ± 0.20	3.08 ± 0.22*
Collagen:				
Hydroxyproline(mg) in whole tissue	4.91 ± 0.49	4.59 ± 0.30	3.95 ± 0.49	2.78 ± 0.26†
Hydroxyproline(mg)/g wet weight	1.24 ± 0.07	1.25 ± 0.07	1.04 ± 0.08	0.90 ± 0.04†
Noncollagen protein:				
Protein(mg) in whole tissue	24.88 ± 4.70	23.37 ± 1.75	27.31 ± 2.62	24.44 ± 2.96
Protein(mg)/g wet weight	6.07 ± 0.91	6.38 ± 0.43	7.28 ± 0.57	7.94 ± 0.78
Collagen/noncollagen protein:				
Hydroxyproline/protein	0.23 ± 0.03	0.20 ± 0.01	0.14 ± 0.01*	0.12 ± 0.01†
DNA:				
DNA(mg) in whole tissue	5.32 ± 0.44	5.04 ± 0.27	5.11 ± 0.30	4.45 ± 0.27
DNA(mg)/g wet weight	1.35 ± 0.04	1.38 ± 0.08	1.37 ± 0.05	1.46 ± 0.05
Prolyl hydroxylase activity:				
dpm × 10^{-6} in whole tissue	4.28 ± 0.42	3.92 ± 0.22	4.09 ± 0.20	2.70 ± 0.26†
dpm × 10^{-2}/DNA(μg)	8.00 ± 0.32	7.83 ± 0.44	8.09 ± 0.40	6.01 ± 0.43†

Each group consisted of 7 rats. Data are shown as means ± SE.
Values are significantly different from the control: * $P < 0.05$. † $P < 0.01$.

Consequently, N-5′ selectively inhibited the accumulation of collagen without changes in noncollagen protein and DNA content in granulation tissue by repeated injections.

These findings were supported by the pathological examination, and a marked reduction of collagen fibers stained blue with Azan-Mallory was observed in granulation tissue treated with 100 mg/kg of N-5′ compared with that of the control group.

Prolyl hydroxylase activity in whole tissue and in DNA (μg) of the tissue was significantly decreased when 100 mg/kg of N-5′ was repeatedly injected (Table 2). Furthermore, N-5′ did not directly inhibit prolyl hydroxylase purified from chick embryo *in vitro* (data not shown). Therefore, these results indicated that N-5′ had decreased prolyl hydroxylase levels in the tissue.

To elucidate the effective phase of N-5′ in carrageenin-air-pouch inflammation, we studied the action of N-5′ by injections at various inflammatory phase. The experimental schedule and results are summarized in Table 3.

When 100 mg/kg of N-5′ was injected immediately after the carrageenin injection and repeatedly injected for 4 days, the selective inhibition of collagen accumulation was observed (Group 1). The effect was shown by treatment with N-5′ in the first 2 days (Group 2), while treatment in the last 2 days did not have any effect (Group 3). These results suggest that N-5′ exerts inhibitory action on collagen accumulation within 2 days after the carrageenin injection.

Effect of N-5′ *on fibroblast proliferation in culture*

The rate of fibroblast proliferation was markedly limited by the addition of 100 μg/ml of N-5′, but the number of cells per plate did not decrease to less than the initial level (Fig. 1).

Effect of N-5′ *on collagen synthesis by diploid fibroblasts*

As *in vitro* system, we used a diploid fibroblast culture from human embryonic skin. This assay system reflected greater normal collagen synthesis *in vivo* as compared with a culture system using lined cells.

Figure 2 indicates that the radioactivities of collagen and noncollagen protein in the medium, the cell layer and both were increased linearly until 9 hr of incubation. After 6 hr incubation, the synthesis of collagen and noncollagen protein was detected with sufficient sensitivity to study the effects of various

Table 3. Effect of various doses of N-5′ on the content of collagen and noncollagen protein in granulation tissue in rats

Day: −1 0 1 2 3 4 (▲ Air (7 ml), ▲ Carr. (4 ml), ▼ Sacri.)	Granulation tissue wet wt (g)	Collagen: Hydroxyprotein (mg)	Noncollagen Protein: Protein (mg)
Control	4.54 ± 0.21 (100)	4.24 ± 0.27 (100)	21.0 ± 0.9 (100)
Group 1	3.90 ± 0.31 (86)	3.08 ± 0.35* (73)	20.9 ± 2.4 (99)
Group 2	3.67 ± 0.36 (81)	2.97 ± 0.37† (70)	17.6 ± 2.1 (84)
Group 3	4.53 ± 0.37 (100)	4.38 ± 0.55 (103)	19.1 ± 2.5 (91)

N-5′ (100 mg/kg, ↑) or dimethylsulfoxide (1 ml/kg, ↑) was injected into the carrageenin-air-pouch according to the experimental schedule shown in the left column. Each group consisted of 7 rats. Numbers shown in parenthesis represent mean percentage of the control. Data are shown as means ± SE. Values are significantly different from the control: * $P < 0.05$, † $P < 0.01$.

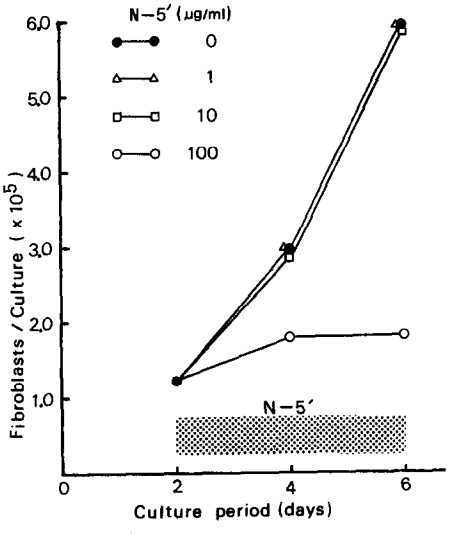

Fig. 1. Effect of N-5′ on human diploid fibroblast proliferation in culture. Human diploid fibroblasts were incubated in 2.5 ml of Dulbecco's modified Eagle's medium containing 5% fetal bovine serum at 37°. After incubation of 2 days, medium was aspirated, and 2.5 ml of fresh medium and 0.1 ml of N-5′ solution (final concentrations: 0 (●), 1 (△), 10 (□) and 100 (○) μg/ml) were added to the culture. The fibroblast count was determined in three cultures by a hemocytometer at the time points indicated.

drugs on collagen synthesis by the incorporation of tritiated proline into the collagen.

As shown in Fig. 3, the ratio of collagen to total protein synthesis tended to be decreased by N-5′ treatment, but the degree of changes was so slight that the significance of the results is not fully recognized at present. In addition, viability of fibroblasts was over 95%, and no cytotoxicity was observed at various concentrations of N-5′ (data not shown). There appears to be no effect of N-5′ on collagen synthesis by fibroblasts *in vitro*.

DISCUSSION

N-5′ has been used as an antiasthma drug, and its pharmacological properties are the inhibition of passive cutaneous anaphylaxis mediated by homocytotropic antibodies and the inhibition of mast cell degranulation [12]. Carrageenin-induced paw edema in rats was slightly inhibited by the oral administration of N-5′ (150 mg/kg) [12].

We studied the effect of N-5′ on carrageenin-air-pouch inflammation as a chronic inflammation model. Topical treatment with N-5′ (100 mg/kg) for 4 days significantly reduced the accumulation of pouch fluid, the number of infiltrating cells and the formation of granulation tissue (Table 1). Anti-inflammatory action of N-5′ was also seen in carrageenin-air-pouch inflammation.

In the whole granulation tissue, the collagen content was dose-dependently decreased by treatment with N-5′ and inhibited by 43% at a dose of 100 mg/kg of N-5′ (Table 2). It is not known whether the selective inhibition of collagen accumulation by N-5′ in granulation tissue was caused by the reduction of pouch fluid and/or cell count, or by the direct action of N-5′ on the granulation tissue.

Fukuhara and Tsurufuji [14] reported the effects of steroidal and nonsteroidal anti-inflammatory drugs on this inflammation model. The results showed that steroidal anti-inflammatory drug strongly inhibited both accumulation of pouch fluid and formation of granulation tissue, while nonsteroidal anti-inflammatory drugs more strongly inhibited the fluid exudation than the granulation tissue formation.

Alvarez *et al.* [22] reported the effects of these drugs on wound healing. Nonsteroidal anti-inflammatory drugs reduced erythema, heat and edema, but did not influence the healing process and collagen synthesis in the wounded dermal portion.

Nakagawa *et al.* [21] indicated that steroidal anti-inflammatory drug inhibited both synthesis of collagen and noncollagen protein in granulation tissue.

Some proteinase inhibitors have also been shown to inhibit granulation tissue formation and reduce both collagen and noncollagen protein content in the tissue by interfering with the initial inflammatory reactions including the migration of PMNs into the inflamed site [23].

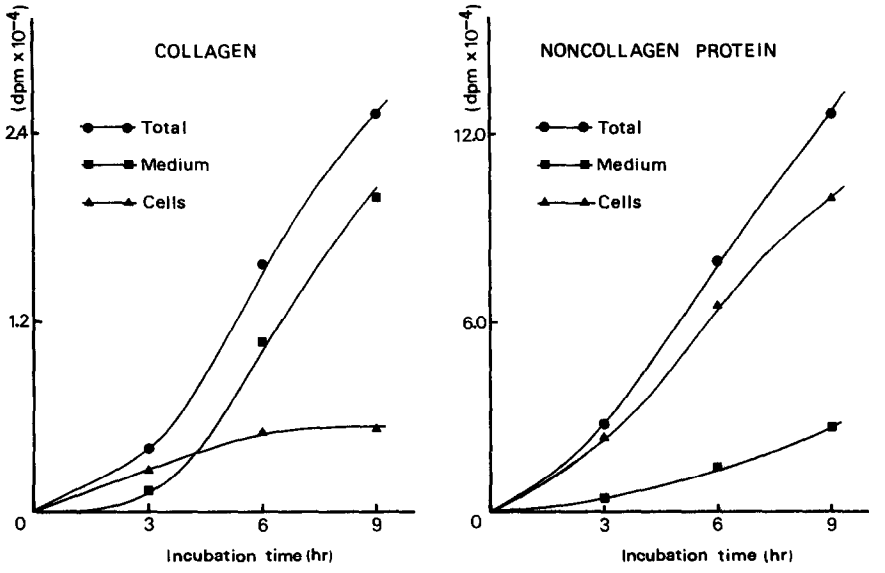

Fig. 2. Time-course of collagen and noncollagen protein synthesis by human diploid fibroblasts in culture. Confluent fibroblasts were incubated in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 50 μg/ml of ascorbic acid for 1 hr and then exposed to 1 μCi/ml of L-(5-^{3}H) proline and 100 μg/ml of β-aminopropionitrile for 3, 6 and 9 hr. The amount of radioactivity (dpm) solubilized by proteinase-free collagenase is a measure of the collagen synthesized, and the radioactivity of the residue represents noncollagen protein synthesized in the medium (■), the cells (▲) and total (●). Each value represents duplicate determination of five cultures.

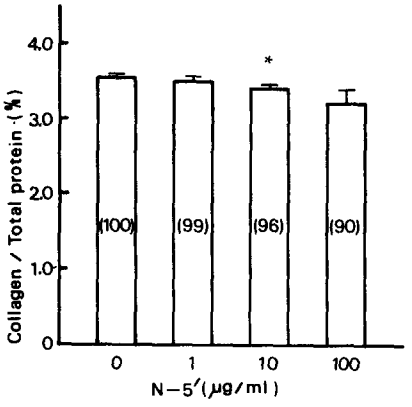

Fig. 3. Effect of N-5′ on the ratio of collagen to total protein synthesis by human diploid fibroblasts in culture. Confluent fibroblasts were incubated with N-5′ (final concentrations: 0, 1, 10 and 100 μg/ml) for 6 hr as described in Materials and Methods. The amount of collagen and noncollagen protein synthesized was determined, and the ratio of collagen to total protein(%) was calculated from [dpm of collagen/(dpm of collagen + 5.4 × dpm of noncollagen protein)] × 100. Numbers shown in parenthesis represent mean percentage of the control, and each column and bracket represent mean of five cultures ± SE. Values are significantly different from the control: * $P < 0.05$.

Consequently, it can be considered that granulation tissue formation and collagen accumulation are regulated by various factors and reactions in the inflammation process. At present, there is no clear similarity of action between N-5′ and drugs such as nonsteroidal anti-inflammatory drugs, steroidal anti-inflammatory drugs and proteinase inhibitors.

The contents of noncollagen protein and DNA in granulation tissue treated with N-5′ were not significantly affected (Table 2), which suggests that the selective inhibition of collagen accumulation did not result from the total depression of granulation tissue formation.

On the other hand, prolyl hydroxylase activity was significantly reduced in whole tissue and in DNA (μg) of the tissue treated with 100 mg/kg of N-5′ (Table 2). Tissue levels of prolyl hydroxylase activity are generally correlated with the rate of collagen synthesis [2] and N-5′ did not directly inhibit prolyl hydroxylase purified from chick embryo *in vitro*.

These results therefore indicate that the reduction of prolyl hydroxylase activity in granulation tissue presumably reflects the reduction of collagen biosynthesis, but it is not clear whether the reduction of prolyl hydroxylase activity was caused by a decrease of fibroblast count, the suppression of collagen synthesis by fibroblasts, or both.

In culture, N-5′ did not exert direct inhibitory action on fibroblast collagen synthesis (Fig. 3), but inhibited fibroblast proliferation (Fig. 1) at a concentration of 100 μg/ml. This would therefore suggest that one of the inhibitory mechanisms of collagen accumulation by N-5′ in inflamed sites involve the direct inhibition of fibroblast proliferation.

Fibroblasts proliferation and/or collagen synthesis have been shown to be affected by various bioactive substances released from inflamed cells such as mast cells [4, 5], macrophages [6, 7], lymphocytes [8, 9]

and platelets [10]. These reports suggest that various inflamed cells act on fibroblasts by releasing chemical mediators and regulate collagen metabolism in the inflamed tissue *in vivo*. Based on this hypothesis, it might be considered that the inhibitory effect of N-5′ on collagen accumulation *in vivo* is caused by the reduction of inflammatory mediators release and thereby the reduction of fibroblast proliferation and collagen synthesis. However, it is not known in any detail at which stage these inflamed cells act and how important this action is in the inflammatory process *in vivo*. Further studies are needed to examine the effects of N-5′ not only on the relationship between collagen accumulation and the release of chemical mediators from mast cells, but also on the function of various inflammatory cells.

In general, various chemical messages are recognized by specific receptors on the cell surface, and each cell's specific functions such as mitogenesis, chemotaxis, and degranulation are performed through the intracellular transmission of biochemical signals [24]. N-5′ is known as an inhibitor of chemical mediator release from mast cells [12] and it has been reported that the inhibitory mechanism involves the inhibition of the energy-requiring system and/or Ca^{2+} influx at the time of degranulation from mast cells [13]. In the case of fibroblast proliferation, Ca^{2+} also plays an important role in the intracellular transmission of chemical messages [25], so that it appears possible that N-5′ acts on the common reaction between mast cell degranulation and fibroblast proliferation.

At present, the exact inhibitory mechanisms of N-5′ on collagen accumulation *in vivo* and fibroblast proliferation *in vitro* are not clear, but further detailed studies of its effects may suggest not only a new pharmacological approach to reduce collagen deposition under pathologic fibrotic conditions but also a mechanism of regulation of collagen accumulation in inflamed tissue.

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