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Effects of Skin Extracts from Arthus Reaction Sites on Collagen Synthesis by Cultured Fibroblasts Derived from Guinea Pig Skin

Atsushi HATAMOCHI, Koji TAKEDA and Hiroaki UEKI

Department of Dermatology, Kawasaki Medical School, Kurashiki 701-01, Japan

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ABSTRACT. Active Arthus reactions were provoked by injecting bovine serum albumin (BSA) into the skin of sensitized guinea pigs. Total and collagenous protein synthesis was determined by treating cultured fibroblasts derived from guinea pig skin with skin extract from the injection sites at 90-minute intervals after challenge injections. The total protein synthesized was determined by the incorporation of radioactive proline, and the collagenous protein synthesized was measured by the incorporation of labeled hydroxyproline. Synthesis of both total and collagenous protein in the presence of the extract from sensitized guinea pig skin was higher than with extract from non-sensitized guinea pig skin. DNA synthesis was not affected by the extract from sensitized guinea pig skin. These findings suggest that collagen synthesis increases in the early phase of an Arthus reaction.

Key words : Active Arthus reaction — Skin extracts — Cultured fibroblasts — Collagen synthesis

The relationship between immunological reaction and metabolism of collagen, which is major protein component in connective tissue, is unknown. In previous study, many authors have reported that factors derived from lymphoid cells stimulate fibroblast proliferation and collagenous and non-collagenous protein synthesis.[1-4] However, it has not been sufficiently investigated how proliferation and collagen synthesis of fibroblasts are influenced by immunologically induced inflammation.

Immune complexes induce a series of inflammatory reactions by activating a complement system. It is very interesting how the reactions influence the metabolism of connective tissue. Recently, we have reported that collagen synthesis increased in the early phase of an Arthus reaction.[5] In this study, we observed the effects of skin extracts from Arthus reaction sites on collagen synthesis by cultured fibroblasts derived from guinea pig skin.

MATERIALS AND METHODS

Induction of Active Arthus Reaction

Active Arthus reactions were provoked by approximately 30 injections of 100 μg BSA in 0.1 ml phosphate buffered saline (PBS) into a 6×6-cm area of the skin on the back of sensitized guinea pigs. Similarly, injections of BSA into the back skin of non-sensitized guinea pigs were performed as controls.

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Preparation of Extracts from Inflamed Skin

At 90-minute intervals after challenge injections, inflamed skin areas were excised, homogenized, defatted and dried with acetone and ether. The dried powders were extracted with PBS at 4°C for 8 hours.[6] Some of them were heat-inactivated at 56°C for 30 minutes prior to use.

Cell Culture

Fibroblast-like cells were isolated from the footpad skin of 50-day-old male albino guinea pigs of the Hartley strain. Skin was excised, immediately washed in PBS supplemented with antibiotics, minced finely, and stirred four times in a preparation of 0.25% trypsin in PBS at room temperature for 20 minutes each time. The fibroblast-like cells were harvested by centrifugation and seeded onto 60×15-mm plastic dishes (Falcon). The preparations were cultured in Dulbecco's modified Eagle's medium (DMEM ; Nissui Seiyaku, Tokyo, Japan) containing glutamine and $NaHCO_3$, and supplemented with 75 IU/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum (FCS ; Flow Laboratories, Rockville, MD, USA). This medium was used as the basic medium for the routine maintenance of the fibroblasts. The cultures were maintained at 37°C in a humidified incubator in 5% CO_2 : 95% air. The medium was changed at 3-day intervals. Routine subcultivation was performed at a 1 : 4 split ratio,[6,7] days after seeding.

In the investigation of the stimulation of DNA and collagen synthesis by skin extracts, 2×10^5 of 10 and 16 population doubling levels (PDL) cells were inoculated into 5 ml basic medium. The medium was changed 4 days after inoculation, and incubation was continued for 6 days, by which time the cell layers had become confluent.

Incubation with Skin Extracts

To study the effect of skin extracts on DNA and collagen synthesis, three dishes for each group were incubated for 4 hours in 5 ml basic medium supplemented with 0.2, 1.0 and 2.0 mg/ml skin extracts.

Measurement of Synthetic Activity of Total and Collagenous Protein

After the incubation of cultures with skin extracts, the medium was removed, and the dishes were washed with basic medium. These cultures were incubated for 4 hours in 5 ml FCS- free DMEM supplemented with 3.4 mg/L α-keto-glutarate Na salt, 50 mg/L ascorbic acid Na, and 5 μCi/ml of L-[3,4-^{3}H]-proline (sp. act., 36.8 Ci/mmol ; NEN chemicals, Boston, MA, USA). At the end of this 4-hour pulse, the medium was transferred to a centrifuge tube, and detached cells and cell debris were removed by centrifugation at 2,000 rpm for 10 minutes. The medium was then dialyzed thoroughly against distilled water at 4°C to remove free proline. BSA was added to a final concentration of 0.02% ; TCA was added to a final concentration of 10%, and the TCA-insoluble precipitates were then collected by centrifugation at 3,000 rpm for 10 minutes.

The cell layer was washed three times with cold PBS and three times with cold TCA, and allowed to dry at room temperature. Four milliliters of 1 N

NaOH was added to each dish, and the preparations were stirred. BSA was added to a final concentration of 0.02% ; TCA was added to a final concentration of 10%, and the TCA-insoluble precipitates were then collected. The precipitates were then hydrolyzed in a sealed pyrex tube with 2 ml of 6 N HCl at 125-135°C for 6 hours. The hydrolysates were dried in vacuo and dissolved in 4 ml of distilled water. Aliquots of 0.1 ml were transferred to counting vials and mixed with 8 ml Aquasol (NEN Chemicals, Boston, MA, USA). The radioactivity then determined was considered a measure of the amount of total protein synthesized. Hydroxyproline-associated radioactivity was assayed in the remaining hydrolysate, according to the method of Juva and Prockop[7] and was considered a measure of the amount of collagen synthesized. The percentage of total protein synthesized as collagen was calculated from the radioactivities in total protein synthesized and in collagenous protein synthesized, according to the assumption of Diegelmann and Peterkofsky.[8]

Measurement of DNA Synthesis

After incubation of the cultures with 2 mg/ml extract in PBS, the medium was removed, and the cultures were washed with basic medium. The dishes were incubated for 3 hours in 5 ml basic medium containing 1 μCi/ml [6-^{3}H]-thymidine (sp. act., 16.2 Ci/mmol ; NEN Chemicals, Boston, MA, USA). At the end of this 3 hour pulse, the radioactive medium was removed, the dishes were washed three times with cold PBS and three times with cold 10% TCA, and fixed in methanol : ethanol (3 : 1). The dishes were then air dried, and the cell layers were dissolved in 1 ml 1 N NaOH. Of this solution, 0.1 ml was diluted to an NaOH concentration of 0.25 N, and 8 ml Aquasol (NEN Chemicals, Boston, MA, USA) was added for the determination of the radioactivity.

Statistical analysis

All data were statistically analyzed with Student's t-test.

RESULTS

Effects of PBS extracts of inflamed skin on total and collagenous protein synthesis by cultured fibroblast-like cells derived from guinea pig skin are shown in Table 1. The incorporation of ^{3}H-proline into total protein increased in the presence of extracts from not only sensitized but also non-sensitized guinea pig skin in proportion to the concentration of the extract. At the concentrations of 0.2 and 1.0 mg/ml, the synthesis of total protein in the presence of extract from sensitized guinea pig skin was nearly the same as with extract from non-sensitized guinea pig skin ; however, at the concentration of 2 mg/ml, the synthesis of total protein in the presence of extract from sensitized guinea pig skin was significantly higher than with that from non-sensitized guinea pig skin. The synthesis of collagenous protein also increased in the presence of extracts from not only sensitized but also non-sensitized guinea pig skin in proportion to the concentration of the extract. At the concentration of 2 mg/ml extract, the synthesis of collagenous protein in the presence of extract from

sensitized guinea pig skin was significantly higher than with extract from non-sensitized guinea pig skin. The synthesis of total and collagenous proteins in the presence of heat-inactivated extracts from sensitized guinea pig skin was also higher than with those from non-sensitized guinea pig skin. The synthesis of DNA was measured in the presence of 2 mg/ml extract in fibroblasts in the stationary phase when the cell layer had become confluent. No increase was shown in the presence of extract from not only sensitized but also non-sensitized guinea pig skin (Table 2).

TABLE 1. Effects of extract with PBS from inflamed skin on total and collagenous protein synthesis by cultured fibroblast-like cells derived from guinea pig skin.

Additions	Incorporation of ^{3}H-proline into total protein total (cell, medium) [10^2 dpm/dish]	Incorporation of ^{3}H-proline into collagenous protein total (cell, medium) [10^2 dpm/dish]	% collagen from total protein [%]
PBS alone	608 ± 17 (407, 201)	175 ± 12 (132, 43)	11.5
Extract from non-sensitized guinea pig skin			
0.2 mg/ml	631 ± 39 (404, 227)	192 ± 21 (135, 65)	12.1
1.0 mg/ml	785 ± 84 (529, 256)	226 ± 6 (182, 60)	11.5
2.0 mg/ml	1027 ± 20 (763, 264)	273 ± 11 (215, 58)	10.6
Extract from sensitized guinea pig skin			
0.2 mg/ml	699 ± 44 (518, 181)	204 ± 5 (148, 56)	11.6
1.0 mg/ml	783 ± 33 (554, 229)	229 ± 18 (165, 64)	11.6
2.0 mg/ml	1348 ± 62(1075, 273)*	448 ± 33 (373, 75)*	13.3
2.0 mg/ml (heat-inactivated)	1241 ± 46(1012, 229)*	412 ± 32 (356, 56)*	13.3

Sixteen PDL were used. Triplicate dishes were determined. Values are expressed as mean ±SE. Difference from extract from non-sensitized guinea pig skin : *$P<0.05$.

TABLE 2. Effects of extract with PBS from inflamed skin on DNA synthesis by cultured fibroblast-like cells derived from guinea pig skin.

Additions	Radioactivity of incorporated ^{3}H-thymidine [10^3 dpm/dish]
PBS alone	48.6 ± 2.0
Extract from non-sensitized guinea pig skin (2 mg/ml)	44.8 ± 1.4
Extract from sensitized guinea pig skin (2 mg/ml)	45.7 ± 2.7

Ten PDL fibroblast-like cells were used. Triplicate dishes were determined. Each value represents the mean ± SEM. There were no statistically significant differences among these data.

DISCUSSION

The data obtained in our previous in vivo experiment[5] indicated first of all that collagen synthetic activity increased in the early phase of the Arthus reaction. It is very important in the examination of the relationship between immunological reaction and connective tissue metabolism to know which factors increase collagen synthetic activity in the early phase of the reaction. Some chemical mediators or infiltrative cells appearing at the sites of the Arthus reaction probably are related to this phenomenon. It has been reported that prostaglandin E_1, E_2[9] and heparin[10] enhance collagen synthesis. We have recently found that histamine also increases collagen synthesis.[11] The data obtained in this in vitro experiment also indicates that collagen synthesis increases in the early phase of the reaction. It is likely that macromolecular components are the factors which increase collagen synthesis in this case, since we conducted dialysis in the PBS extraction procedure. As mentioned above, it is suggested that factors derived from lymphoid cells and macrophages,[12] which play an important role in immunologically induced inflammation, stimulate fibroblast proliferation and collagenous and non-collagenous synthesis. However, in the lesion of the reaction, polymorphonuclear cells which mediate immediate reactions are present initially ; whereas, later, lymphocytes and other mononuclear cells characterizing cell-mediated immunity become the major effectors.[13] It is suggested that serum components exudated in the early phase of the Arthus reaction stimulate total and collagenous protein synthesis of fibroblasts. It is hardly likely that complements stimulate this synthesis, as synthesis also increased in the presence of heat-inactivated extracts.

Among the reports about collagen metabolism accompanying the Arthus reaction, we found only one, that of Nigra *et al.*,[14] which described the changes in the peptidyl proline hydroxylase and collagenase levels in inflamed tissues. They found a clear biophasic response of both enzymes 12 and 96 hours after skin testing. However, peptidyl proline hydroxylase levels do not always correlate with the rate of collagen synthesis.[14] The first peak of enzyme activity corresponds to the period when there is a maximal infiltration of polymorphonuclear cells into the lesion. Previous studies have shown that collagenase is stored in granules in the cells.[15] Presumably the cells could explain in part the origin of collagenase during the initial phase of inflammation.

The data obtained in our previous in vivo experiment[5] also indicated a second increase in collagen synthetic activity 48, 72 and 120 hours after induction of the reaction. This increase suggests that activated-lymphocytes and possibly other mononuclear cells stimulate collagenous and non-collagenous protein synthesis of fibroblasts. There are biophasic increases in not only collagen synthetic activity but also collagenase activity in the reaction site within 120 hours of the induction of an Arthus reaction. We believe that there is a controlled response of connective tissue metabolism in transient inflammation.

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