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Differential effect of etretinate on proliferation and extracellular matrix metabolism of human dermal fibroblasts from elderly and young individuals in a novel three-dimensional culture

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Abstract

The effect of etretinate on proliferation and biosynthesis of collagens and glycosaminoglycans (GAGs) were investigated using human dermal fibroblasts in a novel three-dimensional culture supplemented with L-ascorbic acid 2-phosphate. Fibroblasts from two young and two elderly individuals were studied at different concentrations of etretinate, 0.25, 1.0 and 2.5 μg/ml. Collagens (hydroxyproline) and GAGs (disaccharide units) extracted from the cell layer were analyzed and quantified biochemically by high-performance liquid chromatography (HPLC). Etretinate showed no significant effect on fibroblast proliferation either in the monolayer or the three-dimensional culture. Etretinate increased the collagen or GAGs content in the cell layer, which was prominent at etretinate concentrations of 1.0 and 2.5 or 0.25 μg/ml, respectively in fibroblasts from the elderly ($P < 0.05$). This effect was not seen in dermal fibroblast from the young. These results suggest that etretinate may have the differential effect on collagen and GAG metabolisms depending on the donor age of the cultured fibroblasts. © 1998 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Collagen; Glycosaminoglycans; Aging

1. Introduction

Effects of retinoids on the epidermal cells include terminal differentiation of keratinocytes, basal cell proliferation and normalization of acrofundibular keratinization [1]. Expecting these effects, several retinoids have been widely used in the treatment of abnormal keratinization disorders. Etretinate, an aromatic analogue of retinoic acid, is one of the common therapeutic agents for the treatment of a variety of keratinizing disorders such as severe psoriasis and hereditary palmoplanter keratosis, etc. The skin fragility in psori-

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atic patients under etretinate therapy has been suggested to be of the epidermal origin, but there is a possibility that the extracellular matrix (ECM) of the dermis might be affected by etretinate [2]. It is known that various retinoids modulate extracellular matrix gene expression of the skin [2] and that etretinate reduces the serum level of type I procollagen propeptide in patients with psoriasis as well as in fibroblast culture studies from both patients and healthy subjects [3].

The production of ECM has been reported to be suppressed by etretinate [2–6]. The stimulatory action of etretinate on ECM biosynthesis has not yet been reported. In addition, the previous results of in vitro studies indicated an inhibitory effect on fibroblast growth [5,6].

The present study was aimed to determine the action of etretinate on ECM synthesis by human dermal fibroblasts from the young and the elderly in a novel three-dimensional culture, which can provide more physiological environments for cultured fibroblasts.

2. Materials and methods

2.1. Cell cultures

Human dermal fibroblasts were generated from forearm skin of healthy young (11 and 9 years old) and elderly (67 and 75 years old) individuals after obtaining informed consents. Cells were initiated from explant cultures and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) in an atmosphere of 37°C humidified air and 5% CO_2. At the second passage, fibroblasts were subcultured into three flasks (75 cm^2) and experiments were conducted between three and five passages. Fibroblasts were seeded at 5×10^5 cells/10 cm dish in DMEM supplemented with 10% FCS and 1 mM magnesium salt of L-ascorbic acid 2-phosphate (Asc-2p, Wako Pure Chemical Industries, Osaka, Japan) for 10 days. Ascorbic acid is an essential cofactors for the conversion of proline to hydroxyproline by prolyl hydroxylase. Asc-2p used in this experiments is stable and active for up to 7 days under conventional culture conditions [7]. The supplementation of Asc-2p can render cells to the organization of the self-produced three-dimensional structure during this incubation period [8]. Then the medium was changed to DMEM containing 1% FCS and 1 mM of Asc-2p. Low, middle or high concentrations of etretinate (0.25, 1.0 and 2.5 μg/ml, respectively) was added and cells were further incubated for 1 week. During this incubation period, the same culture medium was changed once. Cells in the control group received no etretinate treatment. At the time of harvest, medium and cell layer were collected separately. The cell layer was rinsed three times with cold phosphate buffer saline, and cut into two equal pieces. One half was used for measurement of hydroxyproline, and the other half was further cut into two equal pieces for measurements of disaccharide units and DNA contents, respectively. All experiments were done in triplicate.

2.2. Etretinate

Etretinate was a generous gift from Hoffman La Roche Company. Stock solutions were prepared in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/ml and stored at −20°C until use. The above indicated doses were freshly prepared from stock solution at the time of addition. Equivalent DMSO was added to the control group. We preliminary confirmed that this concentration of DMSO (0.0125%) was not cytotoxic.

2.3. Quantification of DNA concentration from cultured cells

Genomic DNA was isolated to quantify the DNA content of the cell layer. Briefly, one quarter of the cell layer was treated with lysis buffer containing 100 mM NaCl, 10 mM Tris–HCl, 25 mM EDTA, 0.5% SDS and 0.1 mg of proteinase K and incubated in a water bath at 50°C for 12–18 h. An equal volume of equilibrated phenol and chloroform was added to the cell lysate and centrifuged for 15 min at 7000 rpm. After removal of the top phase, DNA was precipitated by cold ethanol and 7.5 M ammonium acetate. The DNA content was spectrophotometrically measured at

260 nm by Gene Quant RNA/DNA calculator (Pharmacia LKB Biochrom, UK).

2.4. Preparation and measurement of main disaccharide units of GAGs

One quarter of the cell layer was processed for crude GAGs preparation and disaccharide analysis. Isolation of crude GAG and disaccharide analysis was performed by the previously described method [9]. Briefly, the sample of the cell layer was treated with 1% NaOH overnight, neutralized with HCl, digested with pronase, deproteinized with 10% trichloroacetic acid and centrifuged. The supernatant was dialyzed against water and GAGs were precipitated with 0.1% cetylpyridinium chloride in the presence of 0.012 N sodium sulfate. After centrifugation, the precipitate was washed twice with 95% ethanol saturated with NaCl and then with pure ethanol, and dried. The crude GAG was suspended in water and used for further disaccharide analysis. One hundred microliters of sample solutions were evaporated and digested with chondroitinase ABC or with chondroitinase AC II (Seikagaku Kogyo, Tokyo, Japan). Chondroitinase digested disaccharide samples or commercially available disaccharides were labeled with 1-phenyl-3-methyl-5-pyrasolone (PMP) as follows. The samples were dissolved in 0.3 N NaOH and xylose was added as an internal standard. An equal volume of 0.5 M PMP in methanol was added to the solution and the mixture was kept at 70°C for 30 min. After PMP labeling, 0.1 N HCl was added to neutralize and the mixture was extracted with 50 μl of chloroform twice. The aqueous layer was applied to HPLC equipped with a CHEMCO 3-ODS-H column (6 × 100 mm). Elution was performed with a linear gradient of acetonitril/water (3:1 v/v) and 10% of 200 mM phosphate buffer, pH 7.5 at a flow rate of 1 ml/min at 50°C. Peaks were detected at 245 nm. The amount of ΔDi-6S, ΔDi-4S, ΔDi-0S or ΔDi-HA was determined by high-performance liquid chromatography (HPLC) of chondroitinase AC digestion, and the amount of ΔDi-4S(DS) was calculated by deducting the above ΔDi-4S from total ΔDi-4S in the chondroitinase ABC digestion.

2.5. Preparation of insoluble collagen from the cell layer and measurement of hydroxyproline

Half of the cell layer suspended in PBS was sonicated for 5 min at 20 mW by Sonifier 450 (Branson, USA) followed by centrifugation at $10000 \times g$ for 20 min. The supernatant and pellet were collected separately and lyophilized. Quantification of hydroxyproline from the cell layers was performed according to the previously described method [10,11].

3. Statistical analysis

The results were expressed as mean ± S.E. Mean values were statistically analyzed with analysis of variance followed by Fisher's protected least significant differences tests.

4. Results

4.1. Effects of etretinate on human dermal fibroblasts proliferation

Because of the difficulty in trypsinizing fibroblasts into single cell suspension in the three-dimensional culture, DNA content of the cell layer was referred as an indicator for fibroblasts proliferation. Compared with the culture in the absence of etretinate (the control culture), cells from both the elderly and the young subjects revealed no significant changes at different concentrations of etretinate (data not shown). In addition, the etretinate concentrations examined showed no significant effect on fibroblast proliferation in the monolayer culture, either.

4.2. Effects of etretinate on GAGs synthesis

We measured the amount of main disaccharide units of GAG accumulated in the cell layer (Fig. 1). The amount of total disaccharide units from the elderly (75 and 67 years old) increased at 0.25 μg/ml (1.5- and 1.3-fold) and 1.0 μg/ml (1.2- and 1.2-fold), but decreased at 2.5 μg/ml (0.9- and 0.8-fold), respectively. In contrast, the total

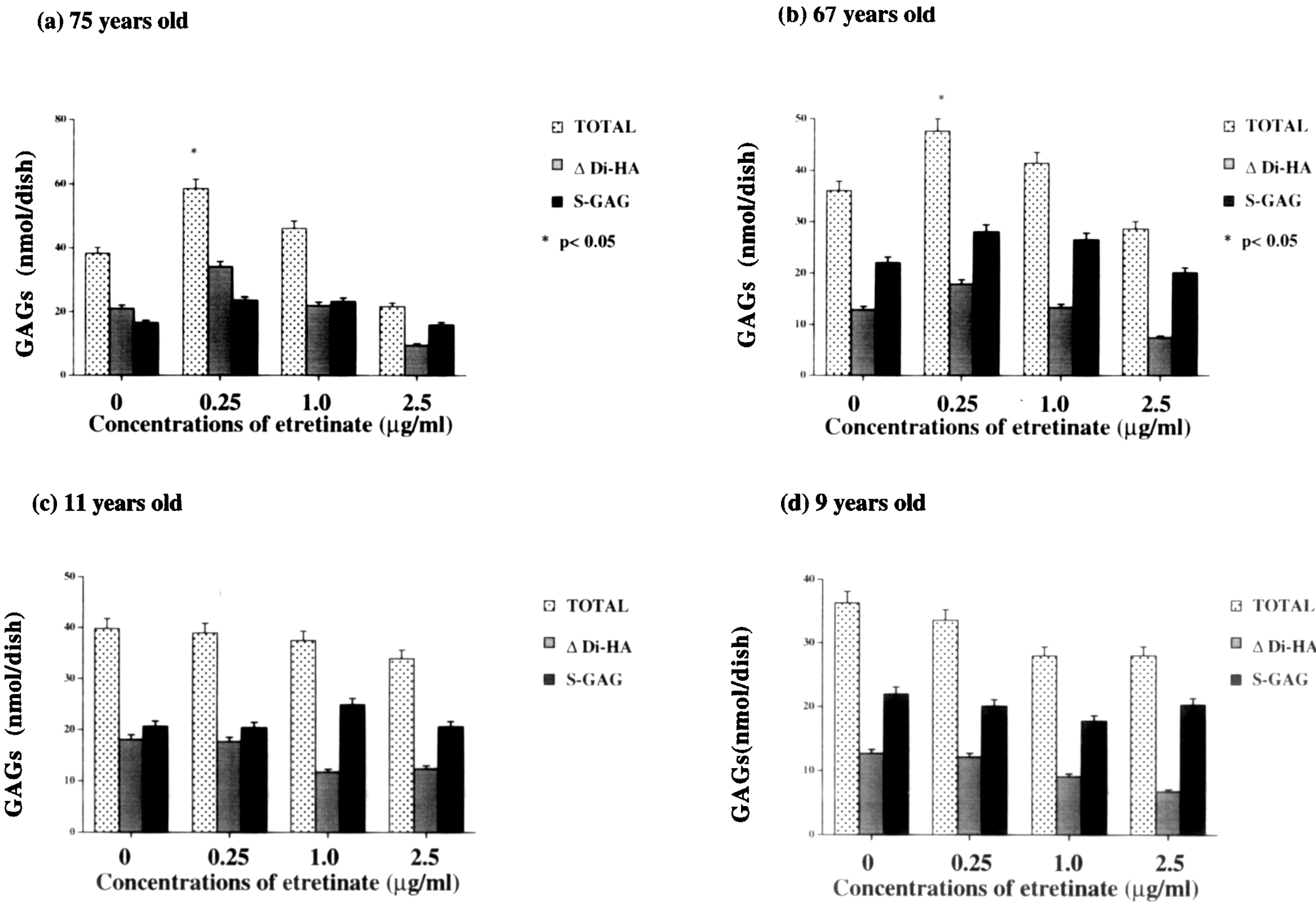

Fig. 1. Effects of etretinate on the GAGs synthesis in three-dimensional culture of fibroblasts from the elderly (a, b) and the young (c, d). Synthesis of GAGs was measured by HPLC after PMP labelling. Values represent are mean ± S.E. of the triplicated samples. ΔDi-HA, disaccharide units of hyraluronic acid; ΔS-GAG, disaccharide units of sulfated GAG. $*P < 0.05$.

Table 1
Effect of etretinate on main disaccharide units of GAGs synthesized by three-dimensional culture fibroblasts from two elderly and two young individuals

Etretinate concentrations (μg/ml)	75 years old		67 years old	
	ΔDi-HA% (mean ± S.E.)	ΔDi-4S (DS)% (mean ± S.E.)	ΔDi-HA% (mean ± S.E.)	ΔDi-4S (DS)% (mean ± S.E.)
0	54.7 ± 4.4	28.9 ± 4.3	35.8 ± 1.3	44.6 ± 1.8
0.25	58.0 ± 3.0	29.0 ± 2.4	36.9 ± 2.6	42.0 ± 2.8
1.0	47.6 ± 2.9	34.2 ± 4.4	31.7 ± 2.6	46.1 ± 2.2
2.5	43.99 ± 0.4	36.6 ± 0.6	25.8 ± 1.3	47.3 ± 3.0
	11 years old		9 years old	
0	45.4 ± 4.1	39.3 ± 4.6	35.1 ± 1.8	45.9 ± 2.5
0.25	45.3 ± 4.2	39.7 ± 4.4	36.0 ± 5.3	45.9 ± 6.6
1.0	34.9 ± 5.0	49.1 ± 4.4	31.7 ± 4.4	50.8 ± 5.4
2.5	38.4 ± 4.6	47.5 ± 6.5	23.99 ± 1.7	61.7 ± 1.8

Disaccharide analysis of cell layer were performed by HPLC from fibroblasts grown in three-dimensional culture treated with etretinate at three concentrations for 1 week.
Values represent mean ± S.E.M. based on triplicated samples. ΔDi-HA% = disaccharide units of hyraluronic acid (%). ΔDi-4S(DS)%-disaccharide units of dermatan sulfate (%).

amount of disaccharide units was unaltered in fibroblasts from the young (11 and 9 years old) at all concentrations examined. No significant changes at any concentrations of etretinate in all four experiments were observed in ΔDi-HA content or Δsulfated-GAG (ΔS-GAG) content that includes chondriotin 4-sulfate, chondriotin 6-sulfate and in particular, dermatan sulfate (DS).

We further analyzed the compositional change in main disaccharide units. Treatment with etretinate altered the proportions of main disaccharide units, i.e. decrease in ΔDi-HA and increase in ΔDi-4S(DS) in a dose-dependent manner, although statistically not significant (Table 1). In fibroblast culture from the elderly (75 years old), ΔDi-HA constituted 54.7 ± 4.4% of the total disaccharide units in the control culture, but decreased to 47.6 ± 2.9% and 44.0 ± 0.4% at 1.0 and 2.5 μg/ml of etretinate, respectively. Etretinate increased the proportion of ΔDi-4S (DS) from 28.9 ± 4.3% in the control culture up to 34.2 ± 4.4% and 36.6 ± 0.6% at 1.0 and 2.5 μg/ml of etretinate, respectively. In fibroblast culture from the elderly (67 years old), ΔDi-HA constituted 53.8 ± 1.3% of the total disaccharide units in the control culture, but decreased to 31.7 ± 2.6% and 25.8 ± 1.3% at 1.0 and 2.5 μg/ml of etretinate, respectively. Etretinate increased the proportion of ΔDi-4S (DS) from 44.6 ± 1.8% in the control group up to 46.1 ± 2.2% and 47.3 ± 3.0% at 1.0 and 2.5 μg/ml of etretinate, respectively. In fibroblasts from the young (11 years old), ΔDi-HA decreased from 45.4 ± 4.1% in the control culture to 34.9 ± 5.0% at 1.0 μg/ml and 38.4 ± 4.6% at 2.5 μg/ml of etretinate. ΔDi-4S (DS) increased from 39.3 ± 4.6% in the control culture to 49.1 ± 7.6% at 1.0 μg/ml and 47.5 ± 6.5% at 2.5 μg/ml of etretinate.

In fibroblasts from the young (9 years old), ΔDi-HA decreased from 35.1 ± 1.8% in the control culture to 31.7 ± 4.4% at 1.0 μg/ml and 23.9 ± 1.7% at 2.5 μg/ml of etretinate. ΔDi-4S (DS) increased from 45.9 ± 2.5% in the control culture to 50.8 ± 5.4% at 1.0 μg/ml and 61.7 ± 1.8% at 2.5 μg/ml of etretinate.

4.3. *Effects of etretinate on collagen synthesis*

Etretinate treatment significantly increased the hydroxyproline content in the cell layer of fibrob-

lasts from the two elderly individuals at 1.0 μg/ml ($P < 0.05$) and from the one elderly individual at 2.5 μg/ml ($P < 0.05$) as compared with the control culture (Fig. 2). Fibroblasts from the young, however, showed no significant change.

5. Discussion

Although the effects of various retinoic acids on the biosynthesis of ECM by fibroblasts have been extensively studied, the results are controversial, especially in in vitro studies [2–5]. Hein et al. [6] reported the reduced collagen production by vitamin A or its derivatives such as etretinate, free acid of etretinate or isotretinoin in a monolayer culture using adult human skin fibroblasts at the concentration of 10^{-6}–10^{-12} mol/l for 5 days incubation. In addition, Edward [12] reported that all trans-retinoic acid treatment had no effect on the total content of the GAGs isolated from the cell layer of monolayer culture and the contracted collagen lattices using human dermal fibroblasts with the addition of all-trans retinoic acid in the concentration of 10^{-5}–10^{-10} mol/l. The experimental conditions employed in these studies such as donor age, origin of the cells, passage numbers, culture medium and concentration of retinoids, are considered to be critical for these conflicting results.

In the present study, we introduced a novel three-dimensional culture to investigate the effect of etretinate on fibroblast proliferation and ECM metabolism in a more suitable condition that mimics dermis. This culture system can provide a more physiological condition for fibroblasts in vitro and enables us to directly measure the accumulated ECM in the cell layer. Morphological and biochemical analyses have documented that collagens and GAGs produced in this culture are similar to those of human dermis [8,9]. The present study showed that etretinate induced the respective changes in collagen and GAG metabolisms of dermal fibroblasts, especially from the elderly; increased production of collagen at 1.0 and 2.5 μg/ml and GAGs at 0.25 μg/ml. It should be noted that the concentration required to increase collagen and GAGs synthesis exists in narrow ranges. Recent observations suggest that the control mechanism of collagen or GAGs acts at the level of mRNA formation. Pharmacological actions of retinoic acid and its analogues are modulated through the nuclear retinoic acid receptor. The formation of a retinoic acid-nuclear receptor complex initiates the further binding to a retinoic acid responsive elements (RARE) and stimulates the transcription of the target gene [1].

Proteoglycans are composed of core protein and side chains of GAG, and work primarily as structural macromolecules maintaining physiological properties of connective tissue [13]. Turnbull et al. [14] revealed the specific inhibition of fibrillogenesis of both type I and II collagens by small DS proteoglycans in bovine tendon. Furthermore, recent studies revealed proteoglycans function as a modulator for growth factors like fibroblast growth factor (FGF) [15] and transforming growth factor-β (TGF-β) [16]. Sato et al. [17] demonstrated the contrary effect of tumor necrosis factor-α on type I and III collagen gene expression by human dermal fibroblasts between the monolayer and the three-dimensional cultures. They also showed that normal dermal fibroblasts gradually decreased gene expression of type I and III collagens in the three-dimensional cultures while they highly maintained the gene expressions in the monolayer culture [18]. Thus, it is feasible that the fibroblast's function or their response to growth factors in the three-dimensional culture is somewhat different from those in the monolayer culture. Our data demonstrated the induction of collagen accumulation as well as substantial increase in GAGs at lower concentration of etretinate along with compositional changes in disaccharide. Thus, etretinate may alter the mutual interaction between collagen and GAGs. Induction of this type of modification may implicate a distinct mechanism by which etretinate affects the quality and the quantity of connective tissue components.

The topical application of all-trans retinoic acid stimulated the deposition of a subepidermal band of collagen in hairless mouse model [19]. Tretinoin emollient cream reduced fine wrinkles and skin roughness, accompanied by histological changes. After 12 months of treatment, there were

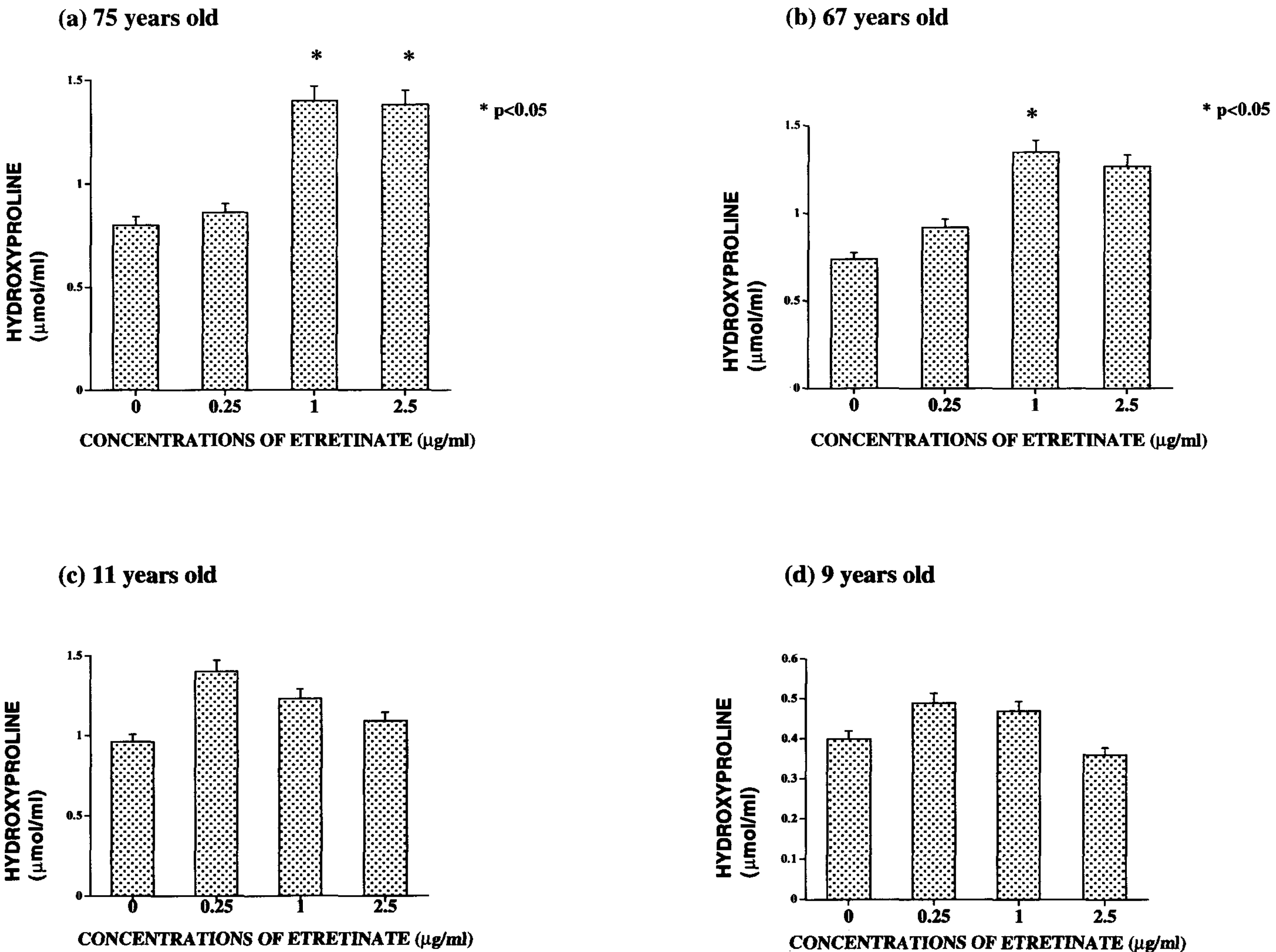

Fig. 2. Effects of etretinate on collagen synthesis in the three-dimensional culture of fibroblasts from the elderly (a, b) and the young (c, d). Content of hydroxyproline from cell layers were measured by HPLC. Values represent are mean ± S.E. of the triplicated samples. $*P < 0.05$.

deposition of new collagen in the papillary dermis and dermal reconstruction with improvement of the dermoepidermal junction [20].

Finally, it should be asked if etretinate has such effects in view of the present observation and if all-trans retinoic acid has similar effects in our experimental system. In order to answer the questions, further in vivo and in vitro studies are required.

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