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Secretion of Non-Helical Collagenous Polypeptides of α1(IV) and α2(IV) Chains upon Depletion of Ascorbate by Cultured Human Cells[1]

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Our previous report showed that human fetal lung fibroblasts secreted non-disulfide-bonded, non-helical collagenous polypeptides of α1(IV) and α2(IV) chains depending on culture conditions [*Connective Tissue* (1999) 31, 161–168]. The secretion of non-helical collagenous polypeptides is unexpected from the current consensus that such polypeptides are not secreted under physiological conditions. The absence of interchain disulfide bonds among α1(IV) and α2(IV) chains was always correlated with the absence of triple-helical structure of the type IV collagen. The finding corresponds with the fact that the interchain disulfide bonds are formed at or close to the completion of the type IV collagen triple-helix formation. The present report shows that ascorbate is the primary factor for the triple-helix formation of the type IV collagen. When human mesangial cells were cultured with ascorbate, only the triple-helical type IV collagen was secreted. However, when the cells were cultured without ascorbate, the non-helical α1(IV) and α2(IV) chains were secreted. Relative amounts of the secreted products were unchanged with or without ascorbate, suggesting that ascorbate is required for the step of the triple-helix formation. The ascorbate-dependency of the triple-helix formation of the type IV collagen was observed in all the human cells examined. The non-helical α1(IV) chain produced by the ascorbate-free culture contained about 80% less hydroxyproline than the α1(IV) chain from the triple-helical type IV collagen. The evidence for the non-association of the non-helical α1(IV) and α2(IV) chains in the conditioned medium was obtained by an anti-α1(IV) antibody-coupled affinity column chromatography for the conditioned medium. Although all the non-helical α1(IV) chains were found in the bound fraction, all the non-helical α2(IV) chains were recovered in the flow-through fraction. The present findings suggest that ascorbate plays a key role in the trimerization step of three α chains and/or in the subsequent triple-helix formation of the type IV collagen.

Key words: ascorbic acid, ascorbic acid 2-phosphate, type IV collagen, triple-helix.

Non-helical type I procollagen α chains are not secreted but retained in the cell, where, until degraded, they interfere with the biosynthetic process, including the transcriptional, translational, and post-translational steps. It is currently accepted that non-secretion and/or degradation may thus provide a quality control system with the type I procollagen (*1–4*). This concept has been extended to other types of collagen including type IV collagen. However, several studies reported that non-helical α(IV) chain(s) was found in conditioned media of cultured cells. These reports discussed that the secretion of the non-helical α(IV) chain(s) is caused by inadequate quality control in the secretory pathway due to abnormal situations such as overexpression of the α(IV) chain(s) (*5*), abnormal secretory apparatus of tumor cells, and repression of hydroxylation by the addition of α,α′-dipyridyl (*6–8*). Most recently, Toth *et al.* reported that several cell-types in culture secreted non-helical α2(IV) chain in association with pro-MMP-9, but not non-helical α1(IV) chain, which remained in the intracellular compartment (*9*).

We previously reported that human fetal lung fibroblasts, TIG-1, secreted non-disulfide-bonded, non-helical α1(IV) and α2(IV) chains into conditioned media (*10*). These were detected by immunoblotting with the monoclonal antibodies specific for the primary sequences of human α1(IV) and α2(IV) chains. The absence of interchain disulfide bonds among the α(IV) chains was correlated with the absence of triple-helical structure of the type IV col-

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Abbreviations: asc 2-p, L-ascorbic acid 2-phosphate; CBB, Coomassie Brilliant Blue; DMEM, Dulbecco's modified Eagle's medium; HASMC, human aortic smooth muscle cells; HMC, human mesangial cells; HUVEC, human umbilical vascular endothelial cells; NC1, non-collagenous domain 1; PBS, Ca^{2+} and Mg^{2+}-free Dulbecco's phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; Tween 20, polyoxyethylene-sorbitan monolaurate.

lagen. Namely, all the non-disulfide-bonded α(IV) chains present in the conditioned media were non-helical in conformation. This happens probably because the interchain disulfide bonds of the type IV collagen are formed at or close to the completion of the triple-helix formation (*10*).

The previous report indicated further that the TIG-1 cells secreted the non-helical α(IV) chains, depending on the culture conditions, particularly in the presence of serum. The requirement of serum for secretion of the non-helical α(IV) chains strongly suggests that the secretion is due not to an intrinsic property of the cells but to disturbance caused by a factor(s) in the serum (*10*). The total contents of α1(IV) or α2(IV) polypeptides (those derived from the triple-helical type IV collagen plus the non-helical chains) in the conditioned media remained essentially unchanged, regardless of the relative amounts of the non-helical α(IV) chains. This implies that post-translational events up to the formation of the triple-helical type IV collagen, rather than the events prior to the translation, are affected by the presence of serum. The serum-dependent secretion of the non-helical α(IV) chains by cultured cells may provide a clue to elucidate a regulatory mechanism of the formation of the type IV collagen triple-helix. Therefore, it is important to elucidate what serum factor or factors cause the secretion of the non-helical α(IV) chains. We here report that the level of ascorbate [ascorbic acid and ascorbic acid 2-phosphate (asc 2-p)] is a key factor for switching between the disulfide-bonded, triple-helical form and the non-disulfide-bonded, non-helical form of polypeptides in the type IV collagen composed of α1(IV) and α2(IV) chains.

MATERIALS AND METHODS

Reagents and Chemicals—Dulbecco's modified Eagle's medium (DMEM) and phosphate-buffered saline (PBS) were purchased from Nissui Pharmaceutical. Penicillin and streptomycin were from Gibco BRL. Trypsin (1:250) was from Difco. Fetal bovine serum was from Cansera International, Canada. L-Ascorbic acid, L-ascorbic acid 2-phosphate magnesium salt, and sodium azide were from Wako Pure Chemical Industries. Polyoxyethylenesorbitan monolaurate (Tween 20), PMSF, and pepstatin A were from Sigma Chemical. Bovine serum albumin (BSA) was from Biogenesis. PVDF membrane was from Millipore. 3,3′-Di-aminobenzidine was from Dojin. Cyanogen bromide–activated Sepharose 4B was from Pharmacia Fine Chemicals. The biotin-conjugated affinity-purified antibody to rat IgG was from Chemicon International. The horseradish peroxidase–conjugated antibody to mouse IgG was from Bio-Rad Laboratories. The horseradish peroxidase–conjugated avidin was from Vector Laboratories. The monoclonal antibody H11, specific for the sequence of human α1(IV) NC1 domain, and H21, specific for the sequence of human α2(IV) NC1 domain, were established as described previously (*11*). The monoclonal antibody JK132, specific for the sequence of human α1(IV) triple-helical domain (*12*), and JK199, specific for the triple-helical conformation of human type IV collagen consisting of α1(IV) and α2(IV) chains (*13*), were obtained from Shiseido Research Center (Yokohama). Type IV collagen of bovine lens capsule was extracted with 0.5 M acetic acid as described before (*14*). All other reagents were of the highest purity.

Cells and Cell Culture—Human mesangial cells (HMC), human aortic smooth muscle cells (HASMC), and human umbilical vascular endothelial cells (HUVEC) were purchased from Clonetics. Fibrosarcoma (HT-1080) and Rhabdomyosarcoma (RD) were obtained from Japan Health Sciences Foundation. Human fetal lung fibroblasts, TIG-1, established at the Tokyo Metropolitan Institute of Gerontology (*15*) were provided by Dr. Kiyotaka Yamamoto.

The cells were cultured in DMEM supplemented with 50 μg/ml of streptomycin and 50 U/ml of penicillin at 37°C under humidified 5% CO_2–95% air. On reaching confluence, the cells were removed from the dish, by treatment with 0.025% trypsin, 0.2 mg/ml EDTA in Ca^{2+}, Mg^{2+}-free PBS, and cultivated at a 1:4 split ratio. For examination of the production of the type IV collagen-related polypeptides, the cells were grown to subconfluence or confluence with 2.5–10% serum-containing media, then culture media were removed. After washing with PBS, the cells were cultured with various concentrations of ascorbate (as asc 2-p or ascorbic acid) in serum-free or 2.5–10% serum-containing media for various periods of days. The conditioned media were collected and subjected to SDS-PAGE or affinity chromatography. In prolonged cultures, the media were replaced every 5 days with 10% serum-containing medium with or without asc 2-p, and the conditioned media were subjected to SDS-PAGE.

SDS-PAGE and Immunoblotting—The conditioned media were mixed with sample buffer: 50 mM Tris-HCl (pH 7.5), 1% SDS, 5% glycerol, and 0.0125% bromophenol blue (final concentrations). 2-Mercaptoethanol (2%) was added for reduction, when necessary. The mixed sample solutions were incubated at 80°C for 5 min, then subjected to SDS-PAGE as described by Laemmli (*16*) with a 1-mm slab gel (3.5% acrylamide for stacking gel and 4.5% acrylamide for separating gel) at a constant current of 5 mA for stacking gel electrophoresis and 20 mA for separating gel electrophoresis. When necessary, the conditioned media were concentrated by ultrafiltration with an Ultrafree-15 Centrifugal Filter Device Biomax-5 (Millipore) at 1,000 ×*g* at room temperature before mixing with sample buffer.

For immunoblotting, the resolved bands on the gel were electrotransferred to PVDF membranes with a semi-dry blot apparatus (Biocraft, model BE-310) at 2.3 mA/cm² for 2 h 15 min as described previously (*12*). All procedures except for blocking were carried out at room temperature. The membranes were incubated with PBS containing 3% BSA overnight at 4°C to block nonspecific adsorption of antibodies. They were then incubated for 3 h with monoclonal antibody JK132, specific for the sequence of human α1(IV) chain triple-helical domain, H11, specific for the sequence of human α1(IV) chain NC1 domain, or H21, specific for the sequence of human α2(IV) chain NC1 domain. The membranes then were washed with PBS containing 0.1% Tween 20 (Tween-PBS). For JK132, the membranes were incubated with a horseradish peroxidase–conjugated antibody to mouse IgG for 2 h at room temperature. For H11 and H21, the membranes were incubated with a biotin-conjugated antibody to rat IgG for 2 h, washed with Tween-PBS, then incubated with a horseradish peroxidase-conjugated avidin for 2 h. After successive washes with Tween-PBS and PBS, the membranes were incubated with 0.6 mg/ml 3,3′-diaminobenzidine and 0.02% H_2O_2 in PBS to visualize the immunoreactivity.

Affinity Chromatography—The monoclonal antibody

JK132 or JK199 was coupled with cyanogen bromide–activated Sepharose 4B. JK132 (44 mg) or JK199 (100 mg) was used with 3.2 g of the dry resin for the coupling reactions, which were carried out according to the manufacturer's instructions. To the conditioned media (100 ml) of HMC cultured for 5 days, the following chemicals were added (final concentrations): 10 mM EDTA, 1 mM PMSF, and 1 μM pepstatin A as inhibitors of endogenous proteases and 0.5 mg/ml sodium azide. The conditioned media cultured without ascorbate and those cultured with 2 mM asc 2-p were applied on a JK132- or JK199-coupled affinity column of 5-ml volume, respectively. After washing with 50 ml of PBS, the bound protein was eluted with 0.2 M glycine-HCl, pH 2.5, containing 0.2 M NaCl. All the procedures were carried out at room temperature.

Determination of Prolyl Hydroxylation Level—Prolyl hydroxylation level was obtained from the contents of proline and hydroxyproline residues determined by amino acid analysis. The non-helical α1(IV) chains were purified by monoclonal antibody JK132-coupled affinity chromatography of conditioned medium from HMC cultured with neither serum nor ascorbate. The triple-helical type IV collagen composed of α1(IV) and α2(IV) chains was purified by monoclonal antibody JK199-coupled affinity chromatography of conditioned medium from HMC cultured without serum and with 2 mM asc 2-p. The purified samples were subjected to SDS-PAGE under reducing conditions, followed by electrotransfer to PVDF membranes. The α1(IV) and α2(IV) chains of bovine lens capsule type IV collagen extracted with acetic acid (*14*) were also separated and electrotransferred in the same way. The membranes were stained with CBB R-250, and the bands corresponding to the non-helical α1(IV) chain and the α1(IV) chain derived from the triple-helical type IV collagen were cut out, washed with distilled water and dried. Each band was treated with 6 M HCl for 72 h at 110°C for gas-phase hydrolysis to amino acids. The PVDF strips were then incubated in 0.1 M HCl/30% (v/v) methanol at room temperature for extraction of the amino acids. The extracts were dried *in vacuo* at room temperature, then subjected to amino acid analysis by HPLC on a reverse-phase column Wakopak WS-PTC (Wako Pure Chemical Industries) using pre-column derivatization with phenyl isothiocyanate (*17*).

RESULTS

Effect of Ascorbic Acid 2-Phosphate on the Secretory Forms of the Type IV Collagen—The conditioned media were collected after culturing HMC for 5 days with DMEM containing 10% serum, then subjected to SDS-PAGE under nonreducing or reducing conditions, followed by immunoblotting. Monoclonal antibodies JK132, specific for the α1-(IV) chain, and H21, specific for the α2(IV) chain, were used for the blotted membranes. For the conditioned medium cultured with 2 mM asc 2-p, both the antibodies reacted with the bands at about 500 kDa under nonreducing conditions (Fig. 1A lane 5 and 1C lane 5). Under reducing conditions, both the antibodies reacted with very closely migrating but separate bands around 180 kDa (Fig. 1B lane 5 and 1D lane 5), suggesting that the 500-kDa bands correspond

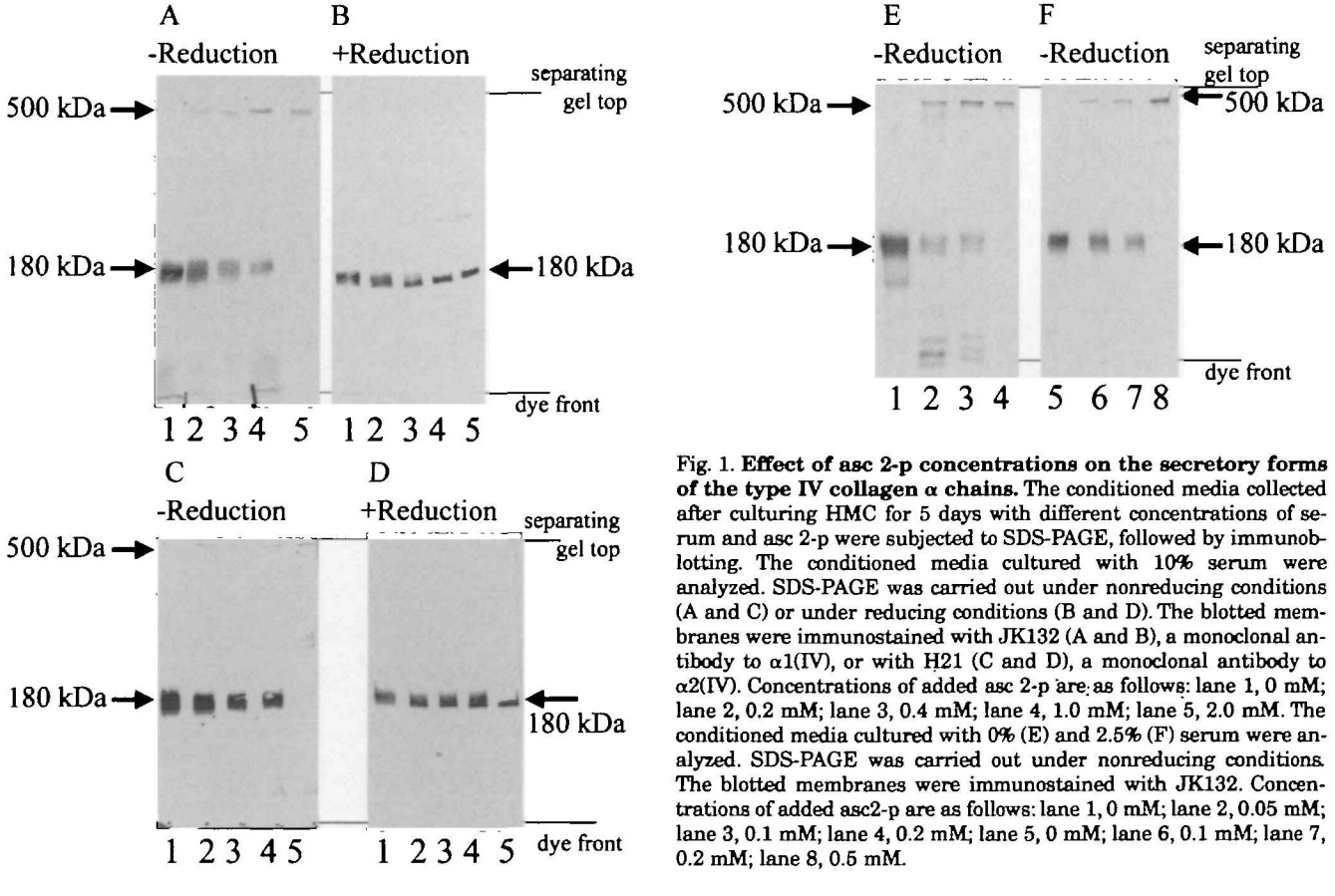

Fig. 1. **Effect of asc 2-p concentrations on the secretory forms of the type IV collagen α chains.** The conditioned media collected after culturing HMC for 5 days with different concentrations of serum and asc 2-p were subjected to SDS-PAGE, followed by immunoblotting. The conditioned media cultured with 10% serum were analyzed. SDS-PAGE was carried out under nonreducing conditions (A and C) or under reducing conditions (B and D). The blotted membranes were immunostained with JK132 (A and B), a monoclonal antibody to α1(IV), or with H21 (C and D), a monoclonal antibody to α2(IV). Concentrations of added asc 2-p are as follows: lane 1, 0 mM; lane 2, 0.2 mM; lane 3, 0.4 mM; lane 4, 1.0 mM; lane 5, 2.0 mM. The conditioned media cultured with 0% (E) and 2.5% (F) serum were analyzed. SDS-PAGE was carried out under nonreducing conditions. The blotted membranes were immunostained with JK132. Concentrations of added asc2-p are as follows: lane 1, 0 mM; lane 2, 0.05 mM; lane 3, 0.1 mM; lane 4, 0.2 mM; lane 5, 0 mM; lane 6, 0.1 mM; lane 7, 0.2 mM; lane 8, 0.5 mM.

to the interchain disulfide-bonded, triple-helical type IV collagen molecule with a chain composition of two α1(IV) chains and one α2(IV) chain (*10*, *13*). When HMC were cultured in ascorbate-free medium, the antibodies reacted with bands of about 180 kDa under nonreducing conditions (Fig. 1A lane 1 and 1C lane 1). Under reducing conditions, the 180-kDa bands remained at the same position. We previously showed that the 180-kDa bands under nonreducing conditions were non-disulfide-bonded, non-helical α1(IV) and α2(IV) chains, and that the absence (or presence) of interchain disulfide bonds among α(IV) chains was always correlated with the absence (or presence) of triple-helical structure (*10*). The 180-kDa bands and the 500-kDa bands decreased and increased, respectively, with increased levels of added asc 2-p (Fig. 1, A and C), indicating that the secretion of the non-helical α(IV) chains increased as the level of extracellular asc 2-p decreased.

Effect of Asc 2-p on the Relative Amounts of the Secreted Polypeptides—The 500-kDa bands derived from the triple-helical type IV collagen molecules under nonreducing conditions did not show the expected amounts of the polypeptides, because the transfer efficiency of the collagenous polypeptides with high molecular weight onto the PVDF membrane was lower and/or the staining of these bands was less reproducible. The total amounts of α1(IV) or α2(IV) polypeptides [the triple-helical type IV collagen-derived α(IV) chain plus the non-helical α(IV) chain] cultured with various concentrations of asc 2-p were semi-quantified from immunostaining intensity at molecular mass of about 180 kDa under reducing conditions (Fig. 1, B and D) and found to be unchanged, suggesting that asc 2-p does not change the total amounts of the polypeptides. Instead, the ascorbate switches the secretory forms of the type IV collagen.

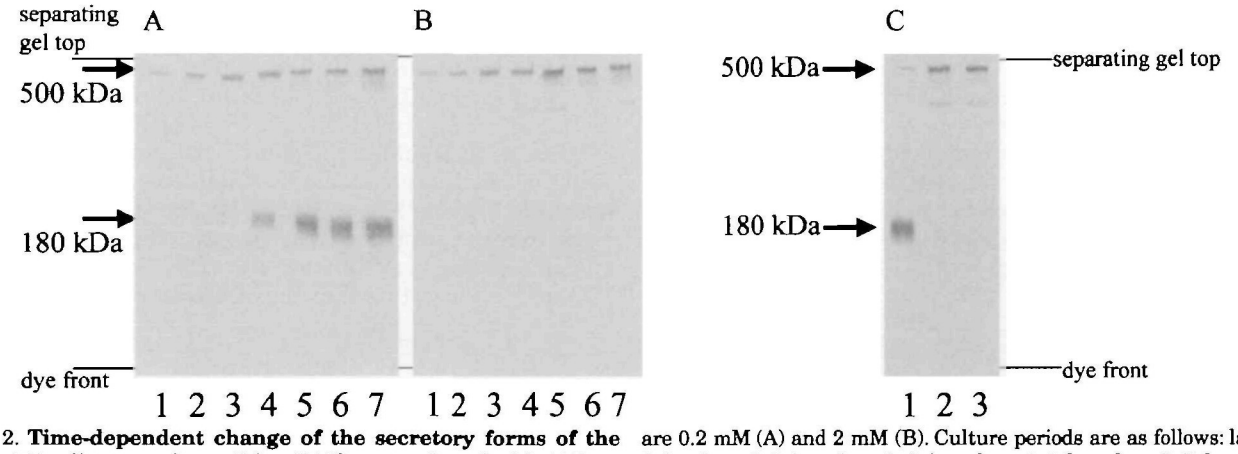

Fig. 2. **Time-dependent change of the secretory forms of the type IV collagen polypeptides.** HMC were cultured with 10% serum for 7 days without replacing the media. The conditioned media were subjected to SDS-PAGE under nonreducing conditions, followed by immunoblotting. The blotted membranes were immunostained with JK132, the monoclonal antibody to α1(IV). An aliquot of the conditioned medium was analyzed daily. Concentrations of added asc 2-p are 0.2 mM (A) and 2 mM (B). Culture periods are as follows: lane 1, 1 day; lane 2, 2 days; lane 3, 3 days; lane 4, 4 days; lane 5, 5 days; lane 6, 6 days; lane 7, 7 days. The conditioned media collected after culturing the cells for 7 days were analyzed (C). Ascorbic acid (0.2 mM) was added once at the start of the culture (lane 1). Either ascorbic acid (lane 2) or asc 2-p (lane 3) was added to the final concentration of 0.05 mM every day.

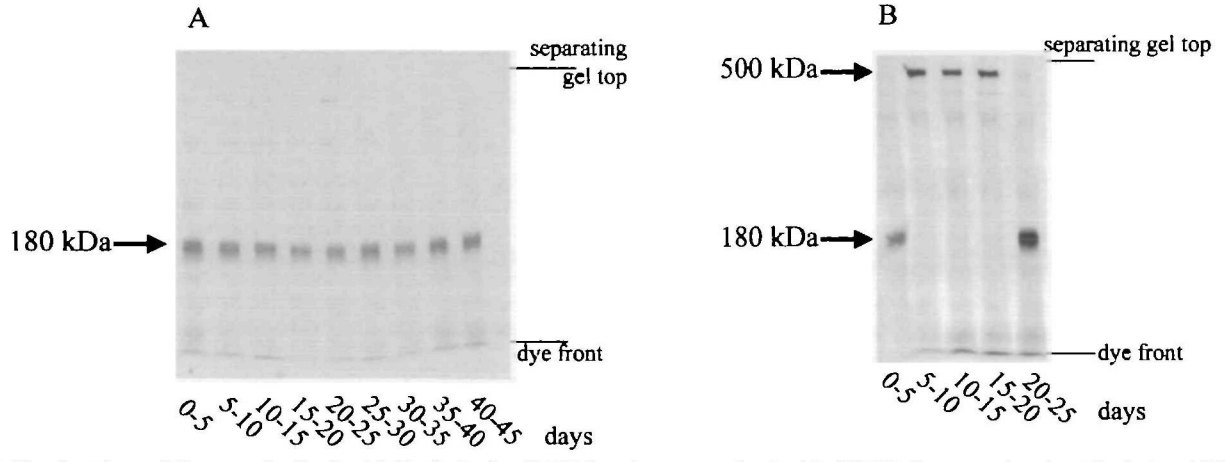

Fig. 3. **Production of the non-helical α1(IV) chain by HMC in prolonged culture.** The conditioned media of HMC cultured with 10% serum were replaced every 5 days with fresh media. The conditioned media were subjected to SDS-PAGE under nonreducing conditions, followed by immunoblotting. The blotted membranes were immunostained with JK132, the monoclonal antibody to α1(IV). The cells were cultured up to 45 days without ascorbate (A). The cells were cultured without ascorbate for the initial 5 days, with 2 mM asc 2-p for 15 days up to 20 days, and without ascorbate for 5 days up to 25 days (B).

Effectiveness of Asc 2-p in the Serum-Containing Media—A higher concentration of asc 2-p was required for production of the triple-helical type IV collagen molecules with higher concentrations of serum in the culture media. When HMC were cultured with 10% serum, 2 mM asc 2-p was required for complete repression of the secretion of the non-helical α1(IV) and α2(IV) chains (no 180-kDa bands), so that all the α1(IV) and α2(IV) chains took the triple-helical conformation (the 500-kDa bands) (Fig. 1A lane 5 and 1C lane 5). But, when cultured with 2.5 and 0% serum, 0.5 and 0.2 mM asc 2-p were sufficient, respectively (Fig. 1F lane 8 and 1E lane 4). To examine the time-dependent change of the secretory forms of the type IV collagen polypeptides, the conditioned medium from the cell culture with an intermediate concentration of asc 2-p was sampled once a day during the culture period. When the cells were cultured with 10% serum and 0.2 mM asc 2-p, only the 500-kDa band was stained at the initial stage of culture. However, the 180-kDa band began to appear from day 3 or 4 (Fig. 2A). When cultured with 2 mM asc 2-p, only the 500-kDa band but no 180-kDa band was stained even after 7 days (Fig. 2B). When cultured with 0.2 mM ascorbic acid, the 180-kDa band was mainly stained after culturing for 7 days (Fig. 2C lane 1). However, when fresh ascorbic acid or asc 2-p was added once a day to a concentration of 0.05 mM, only the 500-kDa band was seen and the 180-kDa band was not detected even after 7 days of culture (Fig. 2C lanes 2 and 3). The data support the notion that the secretion of the non-helical α(IV) chains into the media is caused by depletion of ascorbate.

Production of the Non-Helical α1(IV) Chain by HMC in Prolonged Culture—HMC cultured for a prolonged period (45 days) without ascorbate secreted the non-helical α1(IV) chain (the 180-kDa band) throughout the culture period (Fig. 3A). When the ascorbate-free medium was replaced with 2 mM asc 2-p-containing medium, the conformation of the secreted type IV collagen was switched from the non-helical form to the triple-helical form (the 500-kDa band). Conversely, removal of the ascorbate from the culture medium caused the cells to produce the non-helical type IV collagen (Fig. 3B).

Production of the Non-Helical α1(IV) and α2(IV) Chains by Other Human Cells—Production of α(IV) chains with alternative conformations in response to the level of ascorbate was examined in human cells other than HMC: embryonic lung fibroblasts, TIG-1; aortic smooth muscle cells, HASMC; umbilical endothelial cells, HUVEC; fibrosarcoma, HT-1080; and rhabdomyosarcoma, RD. All the cells secreted the non-disulfide-bonded, non-helical α1(IV) and α2(IV) chains (the 180-kDa bands) alone when they were cultured in ascorbate-free medium. With 2 mM asc 2-p supplemented, these cells secreted the disulfide-bonded, triple-helical type IV collagen molecules (the 500-kDa bands) (Fig. 4, A and B). The results suggest that the ascorbate concentration determines the secretory form of the type IV collagen in human cells that transcribe the type IV collagen genes.

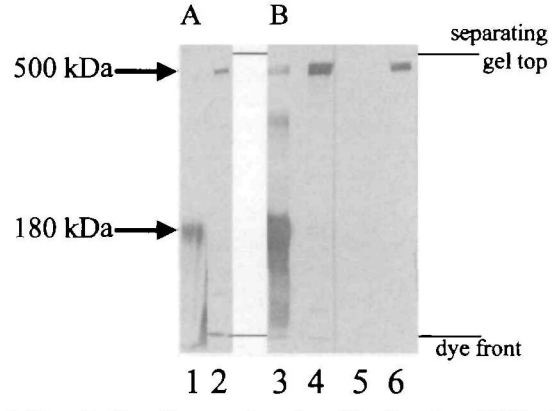

Fig. 5. **Reactivity of monoclonal antibodies to α1(IV) chain.** The conditioned media collected after culturing of HMC for 5 days without serum were subjected to SDS-PAGE under nonreducing conditions, followed by immunoblotting. The blotted membranes were immunostained with the monoclonal antibody to α1(IV), JK132 or H11. A, conditioned media; B, conditioned media 100-fold concentrated by ultrafiltration. Concentrations of added asc 2-p are as follows: 0 mM for lanes 1, 3, and 5; 2 mM for lanes 2, 4, and 6. Lanes 1–4, immunostained with JK132; lanes 5 and 6, immunostained with H11.

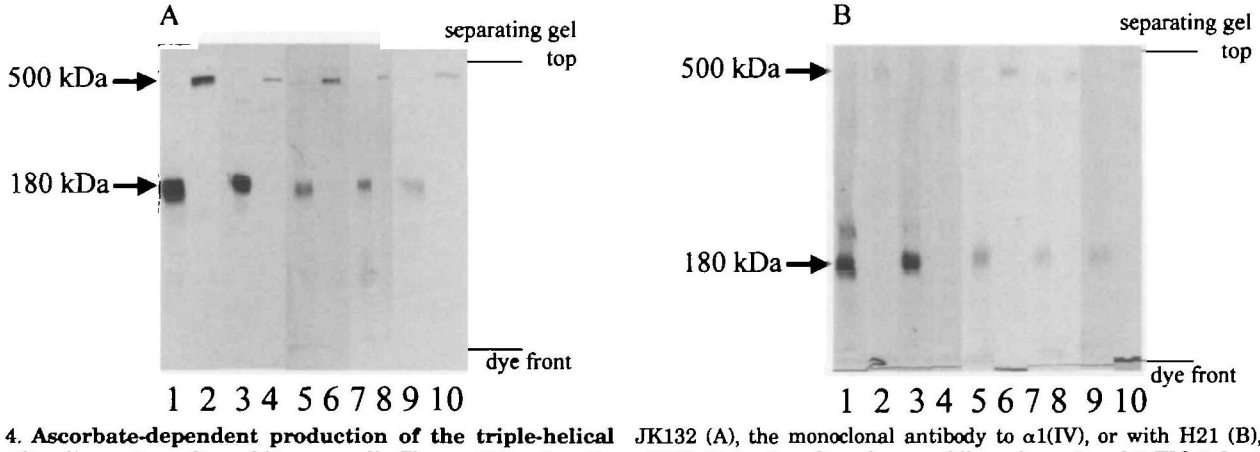

Fig. 4. **Ascorbate-dependent production of the triple-helical type IV collagen by cultured human cells.** The conditioned media collected after culturing cells for 5 days with 10% serum were subjected to SDS-PAGE under nonreducing conditions, followed by immunoblotting. The blotted membranes were immunostained with JK132 (A), the monoclonal antibody to α1(IV), or with H21 (B), to α2(IV). The cells cultured are as follows: lanes 1 and 2, TIG-1; lanes 3 and 4, HASMC; lanes 5 and 6, HUVEC; lanes 7 and 8, HT-1080; lanes 9 and 10, RD. Concentrations of added asc 2-p are as follows: 0 mM for lanes 1, 3, 5, 7, and 9; 2 mM for lanes 2, 4, 6, 8, and 10.

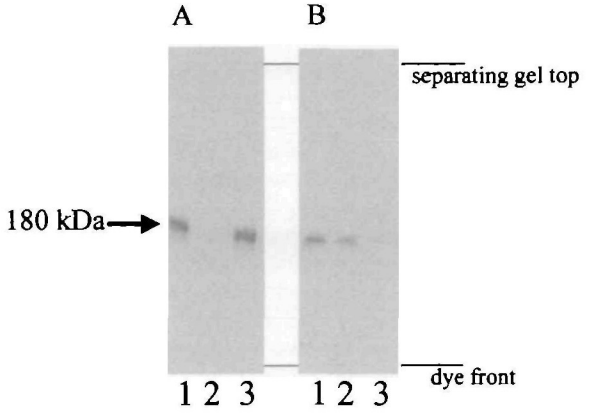

Fig. 6. **Immunoblotting of the fractions from JK132 antibody-coupled affinity column.** One hundred ml of the conditioned medium collected after culturing HMC for 5 days with neither serum nor ascorbate was applied on the JK132 antibody-coupled column. The starting sample (the conditioned medium) (lane 1), the flow-through fraction (lane 2), and the bound fraction (lane 3) were subjected to SDS-PAGE under nonreducing conditions, followed by immunoblotting. The blotted membranes were immunostained with JK132 (A), the monoclonal antibody to α1(IV), and with H21 (B), to α2(IV).

Reactivity of Another Monoclonal Antibody to α1(IV) Chain—Toth *et al.* reported that cultured cells including HT-1080 secreted non-helical α2(IV) chain but not non-helical α1(IV) chain, which remained in the intracellular compartment (*9*). In their study, they used the monoclonal antibody H11, specific for the sequence of human α1(IV) chain NC1 domain (*11*) to detect α1(IV) polypeptide. When we used H11 for immunoblotting of the conditioned media, it reacted only with the α1(IV) chain derived from the triple-helical type IV collagen (the 500-kDa band), and did not react with the non-helical α1(IV) chain (no 180-kDa band). The non-reactivity of H11 with the non-helical α1(IV) chain was confirmed with conditioned media of cultured HMC that was 100-fold concentrated by ultrafiltration (Fig. 5 lanes 5 and 6). On the other hand, the monoclonal antibody JK132 reacted with both the non-helical α1(IV) chain and the α1(IV) chain derived from the triple-helical type IV collagen (Fig. 5 lanes 1–4).

The Non-Helical α1(IV) and α2(IV) Chains Are Not Associated in the Conditioned Medium—The conditioned medium of HMC cultured with neither serum nor ascorbate was applied on a JK132-coupled affinity column. The conditioned medium, the flow-through fraction, and the bound fraction were subjected to SDS-PAGE, followed by immunoblotting with the monoclonal antibody JK132 or H21. All the non-helical α1(IV) chains were recovered in the bound fraction, while all the non-helical α2(IV) chains were recovered in the flow-through fraction (Fig. 6). This indicates that the non-helical α1(IV) and α2(IV) chains were not associated in the conditioned medium.

4-Hydroxyproline Content of the Type IV Collagen α Chains—The non-helical α1(IV) chains were purified from the conditioned medium cultured with neither serum nor ascorbate as the bound fraction on the JK132-coupled affinity column. The triple-helical type IV collagen with the chain composition of two α1(IV) chains and one α2(IV) chain was purified from the conditioned medium cultured with 2 mM asc 2-p without serum by using a JK199-coupled affinity column. The percentage of 4-hydroxyproline in the total imino acid residues (proline + hydroxyproline) was determined from the amino acid analysis. Although 67% of proline residues were hydroxylated in the α1(IV) chain derived from the triple-helical molecule, only 14% of the proline residues were hydroxylated in the non-helical α1(IV) chain (Table I).

TABLE I. **Levels of prolyl hydroxylation in non-helical α1(IV) chains and α1(IV) chains in triple-helical type IV collagen.**

α(IV) chain	4-Hydroxyproline (%)
Non-helical α1(IV) chain	14
α1(IV) chain in the triple-helical molecule	67
α1(IV) chain of type IV collagen from bovine lens capsule	77

Percentages of 4-hydroxyproline in the total imino acid residues (proline + hydroxyproline) were obtained from the amino acid contents determined by amino acid analysis.

DISCUSSION

We previously reported that non-disulfide-bonded, non-helical α1(IV) and α2(IV) chains and disulfide-bonded, triple-helical type IV collagen were secreted under certain cell culture conditions, especially in the presence of serum in the culture medium. The absence of interchain disulfide bonds among α1(IV) and α2(IV) chains was always correlated with the absence of triple-helical structure of the type IV collagen (*10*). The previous report suggested that the secretion of the non-helical α(IV) chains is due to a factor(s) of the extracellular environment rather than intrinsic properties of the cells. The present report shows that ascorbate is the key factor in switching between the triple-helical and non-helical secretory forms of the type IV collagen α chains. The apparent dependency on serum reported previously appears to be caused by repression of the effect of asc 2-p (Fig. 1, A, E, and F). One possible explanation is that serum may contain an enzyme, such as phosphatase, that converts asc 2-p to free ascorbate, which in turn is not stable and cannot function after more than a day of culture (*18*). In the culture with 10% serum, 0.2 mM asc 2-p is sufficient for the synthesis and deposition of the type I collagen (*18*, *19*). However, as described in the present report, the same concentration of asc 2-p is not sufficient for the cells to keep producing only the triple-helical type IV collagen for more than 3 days of culture (Fig. 2A), suggesting that there must be a lower limit (threshold) of ascorbate (asc 2-p and/or ascorbic acid converted from asc 2-p) concentration for producing the triple-helical type IV collagen, and that the ascorbate concentration fell below this level after 3 or 4 days of culture. In response to the lowered level of ascorbate, the cells may have started to produce the non-helical α(IV) chains. The presence of both the 180-kDa and the 500-kDa bands in the conditioned medium may have resulted from production of the 500-kDa polypeptide at an early stage of culture and the 180-kDa polypeptide at a later stage (Fig. 2A). Thus the results can be fully interpreted by positing that the change in ascorbate level is the primary determinant of the conformation of the type IV collagen.

If the ascorbate level in the extracellular environment is directly involved in the events of the type IV collagen pro-

duction inside the cells, it is likely that ascorbate is involved in the process from post-translation to triple-helix formation. The amounts of the α1(IV) and α2(IV) chains in the conditioned media were unchanged regardless of the asc 2-p concentration in the assessment from the immunostaining intensities of the 180-kDa band under reducing conditions (Fig. 1, B and D). This implies that the secretion of the non-helical α(IV) chains was due to the incomplete formation of the triple-helix, not to an imbalance in the translation and secretion of the chains. The simplest interpretation is that the α1(IV) and α2(IV) chains can take two different conformations depending on the level of extracellular ascorbate. We thus postulate as a simplest model for interpreting the present results that ascorbate is involved in the triple-helix formation of the type IV collagen with the chain composition of two α1(IV) chains and one α2(IV) chain. A possible mechanism for the ascorbate involvement in the formation of the type IV collagen triple-helical structure might be worth noting.

The mechanism underlying the triple-helical formation of collagen in general can be mainly classified into two steps, the association of three α chains and the subsequent triple-helical formation. Ascorbate is known empirically to affect the synthesis and deposition of collagen, though the molecular mechanism remains to be elucidated except for the anti-oxidative effect in preventing the oxidation of ferrous ions at the active center of the prolylhydroxylase (*20*). The proline residues residing before glycine need to be hydroxylated for adequate heat-stabilization of the triple-helix conformation. The hydroxyproline content of the non-helical α1(IV) chains was appreciable, amounting to 14% of the total imino acid residues, though much less than that of the α1(IV) chains in the triple-helical molecules (67%) (Table I), suggesting that an adequately high level of hydroxylation is required for the triple-helix formation. It is believed that fibroblasts in culture continue to produce the type I procollagen for a prolonged period without replacement of the medium when 0.2 mM asc 2-p is added in the 10% serum-containing medium (*18*, *19*). If the present finding on the time-dependent production of the non-helical type IV collagen (Fig. 2A) applies to type I collagen, the type I procollagen in the triple-helical conformation would be repressed after 3 days of culture. Whether this happened or not remains to be examined. On the other hand, if the present finding applies only to the type IV collagen, it may mean that the type IV collagen α chain is a poorer substrate for prolylhydroxylase than the type I procollagen α chain.

In the study by Toth *et al.* (*9*), in which non-helical α1(IV) chain was not detected in the conditioned media, the monoclonal antibody H11 was used for the detection. As shown in the results, H11 did not react with the non-helical α1(IV) chain secreted into the culture medium (Fig. 5). The non-helical α1(IV) chain purified by the JK132-coupled affinity chromatography had the sequence of the α1(IV) chain triple-helical region, according to protein sequencing analysis (*10*). Thus the monoclonal antibody JK132 recognizes the non-helical α1(IV) chain. The reason why H11 does not react with the non-helical α1(IV) chain is un-known. The H11 epitope, located close to the C-terminus of the α1(IV) chain (*11*), might be masked by over-modification or might be deleted in the secreted non-helical α1(IV) chain.

The presence of the non-helical α2(IV) chain in the conditioned medium in a comparable amount to but not associated with the non-helical α1(IV) chain (Fig. 6) suggests that the production of the non-helical α(IV) chains could be caused by repressed trimerization in the process of the type IV collagen triple-helix formation. This argument raises the possibility that ascorbate might contribute to correct association of α(IV) chains or stabilization of the associated chains. It is possible that ascorbate activates the apparatus that facilitates the association of the three α(IV) chains. In this respect, it is intriguing to know whether ascorbate affects the activity of HSP47 or PDI, which are putative chaperons in the formation of the collagen triple-helix (*21*, *22*). Little is known about the effect of ascorbate on these chaperons. The repressed trimerization of three α(IV) chains might be related with the loss of reactivity of the non-helical α1(IV) chain to the monoclonal antibody H11.

The effect of asc 2-p on the type IV collagen conformation was observed for all the human cells examined (Fig. 4), suggesting that a general mechanism for the triple-helix formation of the type IV collagen involves the ascorbate level outside of the cells. Alternatively, the ascorbate-dependent triple-helix formation of the type IV collagen might apply to collagenous proteins in general.

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