Type XII and XIV Collagens Mediate Interactions Between Banded Collagen Fibers in Vitro and May Modulate Extracellular Matrix Deformability*

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Type XII and XIV collagens are very large molecules containing three extended globular domains derived from the amino terminus of each α chain and an interrupted triple helix. Both collagens are genetically and immunologically unique and have distinct distributions in many tissues. These collagens localize near the surface of banded collagen fibrils. The function of the molecules is unknown. We have prepared a mixture of native type XII and XIV collagens that is free of contaminating proteins by electrophoretic criteria. In addition, we have purified the collagenase-resistant globular domains of type XII or XIV collagens (XII-NC-3 or XIV-NC-3). In this study, we have investigated the effect of intact type XII and XIV and XII-NC-3 or XIV-NC-3 on the interactions between fibroblasts and type I collagen fibrils. We find that both type XII and XIV collagens promote collagen gel contraction mediated by fibroblasts, even in the absence of serum. The activity is present in the NC-3 domains. The effect is dose-dependent and is inhibited by denaturation. The effect of type XII NC-3 is inhibited by the addition of anti-XII antiserum. To elucidate the mechanism underlying this phenomenon, we examined the effect of XII-NC-3 or XIV-NC-3 on deformability of collagen gels by centrifugal force. XII-NC-3 or XIV-NC-3 markedly promotes gel compression after centrifugation. The effect is also inhibited by denaturation, and the activity of type XII-NC-3 is inhibited by the addition of anti-XII antiserum. The results indicate that the effect of XII-NC-3 or XIV-NC-3 on collagen gel contraction by fibroblasts is not due to activation of cellular events but rather results from the increase in mobility of hydrated collagen fibrils within the gel. These studies suggest that collagen types XII and XIV may modulate the biomechanical properties of tissues.

Type XII and XIV collagens are structurally similar. Both contain a relative short and interrupted triple-helical domain plus three large and apparently identical globular domains at the amino terminus (NC-3 domain), each projecting as an extension of one of the subunit α1 chains. Rotary shadowing images of these globular domains appear as elongated structures, consistent with the predictions from the cDNA deduced sequences (1, 2).

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The Function of Type XII and XIV Collagens

The molecular description of the mechanism by which α2β1 mediates the interaction with collagen fibrils is only partly understood. α2β1 is believed to bind ligands through a tetrapeptide sequence (DGEX) within cytochrome b566 cleavage fragment 3 of the α1 chain of type I collagen (12, 16). In collagen fibrils, these occur along the length of the collagen α chains within the triple-helical domain. This is problematic since the glycine residue is at the center of the triple helix, making the sequence unavailable to the integrin as long as the helical conformation is intact. Binding could occur during “breathing” of the triple helix, but the physical state of the collagen helix during cell binding has not been explored in detail. It is a formal possibility that another molecule may bridge α2β1 and the collagen fibril surface and be responsible for cell binding to collagen fibrils in vivo. Collagens XII and XIV are obvious candidates.

Since type XII and XIV collagens occur in proximity to the collagen fibril surface, either or both could participate in increased integrin binding to the collagen fibril, or in modulating interfibrillar interactions. Therefore, it is represented as a possible system to assess the functional role of type XII and XIV function appeared appropriate. In this study, we have investigated the effect of NC-3 domains of type XII or type XIV collagens, which is expected to influence interactions between fibrils or cells or between fibrils.

Materials and Methods

Purification of Type XII and XIV Collagens and Their NC-3 Domains—Intact type XII and XIV collagens were extracted and purified from fetal bovine skin as described in a previous paper (3, 8). The mixture of type XII and XIV collagens was dialyzed against Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.). After sterilization by filtration, type XII/ XIV collagens in DMEM were stored at -80°C prior to use. The globular domains (collagenase-resistant domains) of type XII and XIV collagens (XII-NC-3 and XIV-NC-3) were extracted and purified from fetal bovine skin as described previously (8). The appropriate Mono Q fractions were stored at -80°C. Protein concentrations were determined using the bicinchoninic acid-based assay according to the manufacturer’s instructions (Pierce Chemical Co.). When added to cells, XII-NC-3 and XIV-NC-3 solutions were dialyzed extensively against DMEM. After sterilization by filtration, XII-NC-3 and XIV-NC-3 solutions were kept at -80°C prior to use.

Cell Culture—Fibroblasts were isolated from human foreskin as described previously (17). The cultures were initiated as out-growths from explants of the skin. The primary cultures were grown in DMEM supplemented with 10% fetal bovine serum (FBS), 2.5 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies, Inc.). Subconfluent cells were dispersed with 0.1% trypsin and 0.02% EDTA in phosphate-buffered saline, pH 7.4 (PBS), and propagated in the above medium. Cultures at the 3rd population doubling were frozen and kept in liquid nitrogen. Foreskin fibroblasts from the 6th-9th population doublings were used throughout these experiments. The cells grown in 10% FBS/DMEM were detached by washing with PBS and subsequently treated with 0.1% trypsin and 0.02% EDTA in PBS. After detachment, trypsin activity was neutralized by the addition of soybean trypsin inhibitor (0.1%). The cells were harvested by centrifugation, resuspended twice in serum-free DMEM, and counted. The amount of pelleted collagen in fibrils was determined using the bicinchoninic acid-based assay according to the manufacturer’s instructions (Pierce Chemical Co.).

Preparation of Collagen Gels—A solution of bovine skin pepsin-digested, collagen fibrils in DMEM was dialyzed against Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.). Subconfluent cells were dispersed with 0.1% trypsin and 0.02% EDTA in phosphate-buffered saline, pH 7.4 (PBS), and 0.02% EDTA in phosphate-buffered saline, pH 7.4 (PBS), and 0.02% EDTA in PBS, phosphate-buffered saline; mAb, monoclonal antibody.

DMEM were added to the collagen solution, and the mixture was warmed to room temperature. Two ml of the solution was added to precoated 35-mm plastic culture dishes, and the collagen was polymerized by incubating the solution for 3 h at 37°C. The peripheral edge of the gel was scraped gently to release them from the dishes, and the collagen gels were allowed to contract uniformly (17).

For centrifugation experiments, collagen solutions were prepared without cells as above. The solutions were then degassed at 4°C by aspiration, and 1 ml of the solution was added to Eppendorf plastic tubes and incubated for 24 h at 37°C.

Measurement of Gel Contraction—The extent of collagen gel contraction by fibroblasts was determined by measuring the diameter of the gels. The area of the gels was calculated from the averaged diameters and expressed as a percentage of the original area. Each data point is an average of measurements from three separate gels. The reproducibility of these experiments is high as reported previously (17). The standard deviations of the means among different series of experiments under the same conditions are usually less than 10%.

Measurement of Gel Contraction by Centrifugation—Gels incubated with or without additions for 24 h at 37°C were centrifuged for 10 min at the indicated relative centrifugal forces. After centrifugation, supernatant solutions were weighed. Collagen gels volume after centrifugation was calculated from the original gel mass (1000 mg: supernatant mass/1000 mg x 100%).

Determination of Cell Numbers Within Collagen Gels—The number of cells in collagen gels was determined by measurement of DNA content as described by Labarca and Paigen (19). Briefly, cells grown in gels were placed in a plastic tube and incubated with 0.2% bacterial collagenase in PBS containing 1 mM CaCl2, for 30 min at 37°C. The floating cells were collected by centrifugation at 300 × g for 10 min. The cell pellets were washed twice with 0.1% trypsin plus 0.02% EDTA in PBS. DNA content was determined fluorometrically.

Cell Attachment Assays—Cell attachment experiments were performed in 24-well plates (Costar, Cambridge, MA). The plastic plates were incubated with XII-NC-3 or XIV-NC-3 in DMEM for 2 h at 37°C and then washed 3 times with DMEM. For cell attachment to type I collagen gels, the plates were incubated with a DMEM solution of type I collagen (1 mg/ml) containing XII-NC-3 or XIV-NC-3 for 2 h at 37°C and then washed 3 times with DMEM. Suspensions of fibroblasts (1.5 × 104 cells/well) in DMEM were incubated for 1 h at 37°C with the indicated additions. The number of attached cells was determined by measurement of DNA as described previously (18). The amount of DNA was converted to cell number by using the factor 8.0 μg of DNA/cell. A collagen gel containing cells was placed in a plastic tube and incubated with 0.2% bacterial collagenase (1 mM CaCl2 in PBS (pH 7.4)) for 2 h at 37°C to dissolve the gels. The floating cells were collected by centrifugation at 300 × g for 10 min. The cell pellets were washed twice with 0.1% trypsin and 0.02% EDTA in PBS. DNA was determined as described by Labarca and Paigen (19).

Heat Denaturation—XII-NC-3 or XIV-NC-3 in DMEM was heated to 60 or 100°C for the indicated times. After heating, the solutions were rapidly cooled on ice. Following denaturation, an aliquot was analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting as described previously (22–23) to assure the presence of intact protein.

Antibodies—Polyclonal antisera to type XII and XIV collagens have been previously described (3, 9). Polyclonal antibodies were purified from rabbit serum by chromatography on protein G-Sepharose. Monoclonal antibodies to type XII and XIV collagens were prepared as described (3, 9). Antibodies were tested for cross-reactivity by enzyme-linked immunosorbent assay (23). The monoclonal antibody to TGF-β1 (TI-21) was obtained from Anogen Inc. (Ontario, Canada) and the monoclonal antibody to the β1 integrin subunit was obtained from Chemicon, Inc. (Temecula, CA).

Recovery of Collagen After Fibril Formation—The collagen gel with or without XII-NC-3 or XIV-NC-3 was incubated at 37°C for 24 h and centrifuged at 15,000 × g for 10 min. The pellets were washed twice with PBS. Following this procedure, aliquots of the XII-NC-3 or the XIV-NC-3, as detected by SDS-polyacrylamide gel electrophoresis and Western blotting, was in the supernatant solutions after centrifugation. The amount of pelleted collagen in fibrils was determined using the bicinchoninic acid-based assay according to the manufacturer’s instructions (Pierce Chemical Co.).

Other Materials—Human recombinant TGF-β1 was purchased from Boehringer Mannheim (Germany). Fibronectin was obtained from Sigma.

1 The abbreviations used are: TGF, transforming growth factor; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; mAb, monoclonal antibody.

2 Formal possibility that another molecule may bridge α2β1 and the collagen fibril surface and be responsible for cell binding to collagen fibrils in vivo. Collagen XII and XIV are obvious candidates.
The Function of Type XII and XIV Collagens

RESULTS

A mixture of type XII and XIV collagens was purified from other components of an extract of fetal bovine skin under non-denaturing conditions as described under “Materials and Methods.” The mixture was judged to be free of contaminating proteins by Coomassie Brilliant Blue staining following gel electrophoresis (data not shown). Unfortunately, we have so far been unable to obtain completely pure preparations of either intact type XII or type XIV alone.

Human fibroblasts were isolated from neonatal foreskin and maintained in culture prior to incorporation into Vitrogen 100 gels (2.5 x 10⁶ cells/ml) as described previously. When the mixture of type XII and XIV collagens was added to the Vitrogen, contraction of the gel by the fibroblasts was significantly increased in a dose-dependent manner (Fig. 1) in the absence of serum. Addition of antiserum to type XII collagen partially reduced the rate of contraction. In order to evaluate this phenomenon more thoroughly, and since we were unable to obtain pure preparations of either intact type XII or type XIV alone, we continued the experimentation with isolated NC-3 domains.

The NC-3 domains of types XII and XIV collagens were isolated from bovine skin and digested with collagenase prior to purification. The resulting products showed only the expected electrophoretic gel bands by Coomassie Brilliant Blue staining (data not shown). There was no reactivity of monoclonal anti-type XII antibody (CLJ) with the type XIV product and no reactivity to monoclonal anti-type XIV (1011G) with the preparation of type XII (data not shown). The rotary shadowed image of the molecules contained in each preparation were examined and found to be identical to those described previously for each collagen type (data not shown).

The addition of the NC-3 domains to Vitrogen solutions had no effect upon the rate of gel formation (data not shown), nor upon the percent of Vitrogen incorporated into the gel (Table I). In the absence of serum and NC-3 domains, the gels contracted minimally (Fig. 2) during 100 h of culture. Addition of the NC-3 domains of type XII and XIV collagens markedly enhanced the rate of gel contraction in the absence of serum in a dose-dependent manner (Fig. 2). The addition of serum (0.3%) or TGF-β1 (10 ng/ml) also stimulated gel contraction in the absence of XII-NC-3 and XIV-NC-3 as expected (Table II). As shown in Table III, the rate of gel contraction XII-NC3 and XIV-NC3 (50 µg/ml) was equal to that caused by serum (0.3%) and was greater than that caused by TGF-β1 (10 ng/ml). When serum or TGF-β1 and XII-NC3 were added simultaneously, the rate of contraction was greater than that that occurred using either agent alone (Table II), suggesting that the mechanism of gel contraction promoted by serum or by TGF-β1 was independent of that of XII-NC3. Essentially identical results were obtained with XIV-NC-3 (data not shown). Further, antibodies to TGF-β1 neutralized the effect of TGF-β1 addition, but had no effect upon the contraction promoting activity of NC-3 domains (Table III).

The gel contraction-promoting activity of XII-NC-3 could be neutralized by the addition of polyclonal anti-type XII collagen antibodies (Fig. 3). The neutralization was antibody concentration-dependent. Addition of anti-type XIV collagen antibodies had no effect upon gel contraction promoted by XII-NC3, nor did anti-type XII antibodies have any effect upon the contraction of gels by type XIV NC3. Anti-type XII antibodies also had no effect upon gel contraction in the absence of any promoting agent nor upon contraction in the presence of serum, indicating that XII-NC-3 is not responsible for the serum effect and confirming previous observations that XII-NC-3 is not present.
XI-NC-3 (50 ng/ml) plus anti-XII polyclonal antibody (A), XII-NC-3 (50 ng/ml) plus anti-XIV polyclonal antibody (B), and anti-XII polyclonal antibody alone (C). Data represent the mean of triplicate determinations and S.D. in normal serum within the limits of detection of Western blotting (data not shown). These data strongly support the hypothesis that the enhancement of gel contraction by type XII collagen NC-3 is specific to that molecular domain and is not due to minor contaminants in the preparation.

Gel contraction enhancement by type XII or type XIV collagens requires the native conformation of the NC-3 domain.

**TABLE II**

Effects of XII-NC-3, FBS, or TGF-β1 on collagen gel contraction

Data represent the mean of triplicate determinations and S.D.

|        | FBS | TGF-β1 | XII-NC-3 | Percent of original area on day 4 |
|--------|-----|--------|----------|----------------------------------|
| %      | ng/ml | μg/ml | %        |                                   |
| 0      | 0     | 0      | 0        | 94.0 ± 1.5                       |
| 0      | 0     | 5.0    | 0        | 66.2 ± 2.6                       |
| 0      | 0     | 50     | 0        | 29.0 ± 1.3                       |
| 0.3    | 0     | 50     | 0        | 27.5 ± 1.5                       |
| 0.3    | 0     | 50     | 0        | 13.2 ± 0                         |
| 0      | 0.1   | 0      | 0        | 70.6 ± 2.1                       |
| 0      | 0.1   | 5.0    | 0        | 45.2 ± 1.7                       |
| 0      | 10.0  | 0      | 0        | 44.5 ± 2.6                       |
| 0      | 10.0  | 50     | 0        | 22.7 ± 1.3                       |

**TABLE III**

Effect of mAb TGF-β1 on collagen gel contraction by promoting factors

Data represent the mean of triplicate determinations and S.D.

| Promoting factor | mAb TGF-β1 | Percent of original area on day 4 |
|------------------|------------|----------------------------------|
| %                | μg/ml      |                                    |
| None             | 0          | 92.9 ± 3.1                        |
| TGF-β1 (10 ng/ml)| 0          | 40.5 ± 0                          |
|                  | 0.1        | 43.7 ± 2.2                        |
|                  | 1.0        | 82.7 ± 0                          |
|                  | 5.0        | 89.8 ± 2.5                        |
| XII-NC-3 (50 μg/ml) | 0          | 26.6 ± 2.6                        |
|                  | 5.0        | 25.8 ± 1.5                        |
| XIV-NC-3 (50 μg/ml) | 0          | 27.3 ± 1.7                        |
|                  | 5.0        | 26.6 ± 2.6                        |
| FBS (0.3%)       | 0          | 28.2 ± 1.9                        |
|                  | 5.0        | 35.0 ± 2.0                        |

**FIG. 4.** The effect of XII-NC-3 and XIV-NC-3 on collagen gel contraction requires native protein structure. A, XII-NC-3 and XIV-NC-3 were incubated at 60 °C for 5 min (■), 15 min (□), or 30 min (▲), or at 100 °C for 15 min (●) or 30 min (▲). The effects of heated XII-NC-3 or XIV-NC-3 (50 μg/ml) were compared with control (□) and native XII-NC-3 or XIV-NC-3 (▲). The gel contraction was determined on day 4. Data represent the mean of triplicate measurements and S.D. B, XII-NC-3 or XIV-NC-3 after incubation at 60 °C for 0 min (lanes 1 and 5), 5 min (lanes 2 and 6), 15 min (lanes 3 and 7), and 30 min (lanes 4 and 8) were separated by SDS-polyacrylamide gel electrophoresis on a gradient gel from 3 to 5% and analyzed by Western blotting with polyclonal antibody XII (lanes 1–4) and polyclonal antibody XIV (lanes 5–8).

Incubation of XII-NC-3 or XIV-NC-3 at 60 °C for 5–30 min, or 100 °C for 15 min causes a significant loss of activity (Fig. 4A). The loss of activity is not due to degradation of the proteins during the incubation since intact XII-NC-3 and XIV-NC-3 were recovered at the end of the incubation period with little or no loss as judged by immunoblotting following electrophoresis (Fig. 4B). Heating the materials caused some loss of interchain disulfide bonding with increased incubation time, particularly with type XIV NC-3 (Fig. 4B, lanes 6–8).

The XII-NC-3 domain does not have to be incorporated into the gel during fibril formation in order to enhance gel contraction. As shown in Fig. 5, the addition of the NC-3 domain to the medium overlaying the gel causes a rate of contraction equivalent to that seen when XII-NC-3 is added at gel formation. Identical results were obtained with XIV-NC-3 (data not shown).

The mechanism by which types XII and XIV collagen NC-3 domains promote gel contraction is not clear from the studies described above. Therefore, further studies were conducted to evaluate three possible mechanisms. Gel contraction could be promoted by (a) an effect upon the cells to up-regulate integrin α2β1 either by an increase of integrin upon the cell surface or by an increase in cell number; (b) NC-3 domains increasing the interaction of cells with fibrils by binding the cell through a receptor, possibly α2β1 or another integrin, and binding to the fibril surface; (c) NC-3 domains decreasing the resistance to contraction by minimizing interfibrillar interactions, allowing individual fibrils to slide past one another.

The gel contraction by fibroblasts is known to depend on the number of cells within the gel. In the absence of additions, no
promotes during gel formation of triplicate determinations and S.D. gel contraction was observed by 2.5 x 10^4 cells/ml as used in all experiments reported here. However, fibroblasts at 10 x 10^4 cells/ml caused gel contraction equivalent of that seen using 50 μg/ml of NC-3 domains (data not shown). The NC-3 domain of type XII collagen has no effect upon the proliferation rates of cells grown in collagen gels as shown in Table IV. Even in the presence of 0.3% serum, no proliferative effect was observed. Similar results were obtained with XIV-NC-3 (data not shown). Therefore, the NC-3 domains do not have mitogenic effects nor are the preparations measurably contaminated with mitogens.

As shown in Fig. 6A, mAb to the β1 integrin chain completely inhibited collagen gel contraction by fibroblasts, irrespective of whether the contraction experiments were performed in medium containing 0.3% FBS, in medium containing 50 μg/ml XII-NC-3, or in the presence of larger numbers of cells (10 x 10^4 cells/ml; data not shown). These results indicate that fibroblasts in serum-free condition express a collagen receptor, possibly α2β1 or other β1 integrins, and that β1 integrins are necessary to transduce intracellular forces to the extracellular matrix. The NC-3 domains have no effect upon gel contraction in the absence of the cell-mediated contractile forces.

Culturing fibroblasts in collagen gels causes the cells to assume an elongated bipolar spindle-like shape. It is known that the process of gel contraction correlates to the elongated shape of fibroblasts (17, 24). Morphology of fibroblasts in collagen gels was examined after culture for 24 h. As shown in Fig. 6B, fibroblasts in collagen gel showed an elongated shape in DMEM (serum-free), in DMEM containing XI-NC-3 (50 μg/ml), and in 0.3% FBS/DMEM. However, fibroblasts became spherical in the presence of mAb β1 integrins (0.5 μg/ml), suggesting that cell elongation requires integrins. In contrast, polyclonal anti-type XII antibodies, which inhibit the gel contraction promoted by XII-NC-3, had no effect upon the elongated shape of fibroblasts in the gel. The results suggest that anti-type XII-NC-3 antibodies do not interfere with the attachment of cells to fibrils.

Neither XII-NC-3 nor XIV-NC-3 promotes fibroblast attachment to plastic or gel-covered substrates. As shown in Fig. 7, neither NC-3 domain has any effect upon fibroblast attachment to a collagen gel. Surprisingly, both domains decreased cell binding to plastic substrates in a concentration-dependent manner. Our interpretation of this result is that the NC-3 domains are not ligands of high affinity for fibroblast cell surface binding proteins and that coating plastic with these domains decreases the surface area of the substrate that is permissive for fibroblast attachment. However, these domains have no effect on the fibroblast attachment to collagen fibrils. We be-

| FBS | XII-NC-3 | Cell number (10^4 cells) |
|-----|----------|-------------------------|
| %   | μg/ml    |                        |
| 0   | 0        | 4.7 ± 0.3               |
| 10  | 10       | 4.2 ± 0.3               |
| 50  | 50       | 4.4 ± 0.5               |
| 0.3 | 0        | 4.6 ± 0.2               |
| 10  | 10       | 4.3 ± 0.2               |
| 50  | 50       | 4.0 ± 0.1               |
| 1.0 | 0        | 7.3 ± 0.4               |
| 10  | 10       | 7.6 ± 0.4               |
| 50  | 50       | 8.1 ± 0.1               |

The Function of Type XII and XIV Collagens

![Figure 5: Addition of XII-NC-3 after collagen gel formation promotes collagen gel contraction. XII-NC-3 (50 μg/ml) was added during gel formation (●) or added to the formed gel at 3 (▲) or 42 h (■) after gel formation. Control culture (○). Each point represents the mean of triplicate determinations and S.D.](image)

![Figure 6: Inhibition of collagen gel contraction and cell elongation by monoclonal anti-β1 chain of integrins antibody. A, the mAb was added at the indicated concentrations and the contraction was determined on day 4. Effects of anti-β1 integrin antibodies on gel contraction in the presence of XII-NC-3 (50 μg/ml) (●), and 0.3% FBS (○). Data represent the mean of triplicate determinations and S.D. B, morphological changes of fibroblasts cultured in a gel for 24 h under control conditions (serum-free) (a), or with the addition of 0.5 μg/ml mAb β1 integrin (b), 50 μg/ml XII-NC-3 (c), 50 μg/ml XII-NC-3, and 0.5 μg/ml mAb β1 integrin (d), and 50 μg/ml XII-NC-3 and 50 μg/ml polyclonal antibody XII (e).](image)
believe that this reflects a low affinity of the NC-3 domains for fibrils relative to their affinity for plastic substrates.

The binding of the NC-3 domains of type XII and XIV collagens to monomeric and fibrillar collagen was then evaluated by enzyme-linked immunosorbent assay. No specific binding to type I, II, or III collagen monomers or fibrils was observed (data not shown), confirming previous observations (10). Binding of the NC-3 domains to collagen fibrils was then evaluated. Vitrogen gels were cast in the presence or absence of type XII or XIV NC-3 domains (Fig. 6). The supernatant solutions were then qualitatively assayed for the amount of NC-3 domains by Coomassie Brilliant Blue staining and immunoblotting relative to the initial amount added. More than 95% of the NC-3 domains were recovered from the supernatant solutions. It is possible that the remaining 5% of the NC-3 domains bound the fibrils but that concentration of NC-3 has minimal effect upon gel contraction. The results indicate that the NC-3 domains do not significantly partition to the fibrillar phase, supporting the previous indications that these domains appear not to bind the fibril surface.

Although type XII and XIV collagen NC-3 domains appear not to bind the fibril surface, we observed a marked effect upon the deformability of Vitrogen gels. As seen in Fig. 8A, NC-3 of both type XII and XIV collagen significantly decreased the relative centrifugal force required to compress the gel. Bovine serum albumin, TGF-β1, and fibronectin had no effect. This change in the physical properties of the collagen gel was dependent upon the native structure of the NC-3 domains (Fig. 8B), as it was abolished by heating the domains. Also, anti-type XII antibodies neutralized the effect observed with XII-NC-3. Thus, gel compression is inhibited by the same manipulations that inhibit gel contraction. Intact type XII and XIV were also evaluated under these conditions (Fig. 8C) with identical findings. The results suggest that contraction promotion by NC-3 domains is not due to cellular events but rather results from modulation of interfibrillar forces.

Since collagen gels made from acid-soluble collagen (which retain both telopeptides) have greater stiffness and are more translucent than those made for the same concentration of Vitrogen (pepsin-solubilized collagen lacking telopeptides) and since acid-soluble collagen fibrils more closely resemble collagen fibrils made in vivo, we evaluated both fibroblast-mediated gel contraction and centrifugation-driven gel compression using acid-soluble collagen gels. The effects of NC-3 domains were qualitatively identical to those seen using Vitrogen gels (data not shown). The amplitude of the changes observed were somewhat less, probably due the increased stiffness of the acid-soluble collagen-derived gel.

**DISCUSSION**

In these studies, we have evaluated the observation that the NC-3 domains of type XII and XIV collagens promote the contraction of collagen gels. We believe that gel contraction is a suitable model system for studying the interaction of mesenchymal cells with a fibrous collagen substrate. We have assumed that the contraction phenomena are a dynamic equilibrium between cell-derived forces originating within the actin bundles and transmitted to the matrix through transmembrane binding proteins and the resistance of the matrix to deformation. We have been unable to demonstrate any direct stable interaction of the NC-3 domains with skin fibroblasts, with monomeric fibrillar collagens, or with collagen fibrils. If centrifugal compression is substituted for the cell-derived forces that drive contraction, the NC-3 domains promote gel compression. The sensitivity of this phenomenon is directly comparable with that of gel contraction with regards to inhibition by antibodies and NC-3 conformation dependence. Our results indicate that the contraction promotion observed can be explained by an effect of the NC-3 domains upon the resistance of a collagen matrix to applied force, and appears not to modulate cell-associated events. The effect is specific to these collagen types since it is inhibited by well characterized antibodies.

**Fig. 8.** XII-NC-3, XIV-NC-3, and intact type XII and XIV facilitate the deformability of the collagen gel by centrifugation in the absence of cells. A, the effects of XII-NC-3 (●) and XIV-NC-3 (■) (50 μg/ml) were compared with those of control (○), 50 μg/ml bovine serum albumin (□), 10 μg/ml TGF-β1 (△), 0.3% FBS (▲), and 50 μg/ml fibronectin (□). B, the effects were abolished by heating of XII-NC-3 (○) and XIV-NC-3 (□) (50 μg/ml at 60 °C for 30 min) as compared with XII-NC-3 (●) and XIV-NC-3 (■) (50 μg/ml). Anti-XI1 antibodies (50 μg/ml) (●) inhibited the effect of XII-NC-3. Anti-XII antibodies (50 μg/ml) (■) had no effect. C, the effect of the deformability of the gel. 50 μg/ml type XII and XIV collagens (○) were compared with 50 μg/ml XII-NC-3 (▲) and to untreated control (×). In A–C, each point represents the mean of triplicate determinations and S.D.
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The addition of other matrix molecules such as chondroitin sulfate or hyaluronic acid have no effect upon the system (25), while heparin has an unexplained inhibitory effect.

The NC-3 domains do not effect the polymerization of collagen monomers into fibrils, nor do they cause concentration of fibrils by dehydration since the addition of the domains to solutions overlying gels has no effect in the absence of forces generated either physically or biologically. The simplest explanation of the effects of NC-3 addition is that these domains decrease interactions between collagen fibrils. Theoretically, such forces could be minimized if the domains decrease ionic or hydrophobic bonding at the sites on the fibril surface where fibrils intersect within the gel. This could be accomplished if the NC-3 domains compete for the interfibrillar interaction sites, thereby decreasing bonding between adjacent fibrils. It could also occur by an increase in the effective fibril diameter since in a fibril solution with constant collagen concentration, an increase in diameter results in a decrease in fibril surface area and number of interaction sites among fibrils. Interfibril interactions would be predicted to vary directly with fibril surface area and number of the sites.

While we believe that this gel contraction system is a valid model for the evaluation of the interaction of fibroblasts with collagenous substrata, its relationship to physiological events is less clear. Fibroblasts placed in a fibrous but acellular environment of the vitreous humor of the eye will cause contraction of that gel, similar to what is observed in vitro (26). Therefore fibroblasts can recognize and reorganize collagen fibers in vitro despite the increased complexity of the biological matrix. The ability of type XII and XIV NC-3 domains to modulate interfibrillar interaction of these more complex fibrillar systems is not known. However, the dramatic in vitro results strongly support the hypothesis that types XII and XIV mediate biomechanical aspects of the matrix.

The results shown here are consistent with the possibility that both type XII and XIV collagens participate in extracellular matrix deformability. By decreasing interfibrillar interactions, the matrix could become locally more pliable in the absence of cellular stress, or could become progressively more rigid if the NC-3 domains facilitate cell-mediated alignment and concentration of banded collagen fibrils. This possibility might resolve the apparently contradictory finding that types XII and XIV are associated with both large and small-diameter collagen fibrils (9, 27, 28). The model we propose assumes that the outcome of the association of types XII and XIV with the banded fibrils is dependent upon the local cell activity. There is some experimental support for this concept. Schafer et al. (29) observed that fibroblasts derived from the papillary dermis were less able to contract collagen gels than fibroblasts from the reticular dermis. In addition, mixing papillary cells with reticular cells inhibited gel contraction. These data suggest that the greater fibril density of the reticular dermis may be due to the increased activity of the reticular cells. Thus, it is possible that in concert with appropriate local cellular activity, types XII and XIV could mediate increased or decreased matrix deformability.

These studies show no differences between the effect of type XII and of type XIV. This is surprising since the two molecules segregate to nearly exclusive tissue regions in vivo (9). It is possible that the effects of either XII or XIV collagens could be modulated differentially in vivo by the presence of the larger transcript in the case of type XII or by the addition of glycosaminoglycan.

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REFERENCES

1. Yamagata, M., Yamada, K. M., Yamada, S. S., Shinomura, T., Tanaka, H., Nishida, Y., Obara, M., and Kimata, K. (1991) J. Cell Biol. 115, 209-221
2. Gerecke, D. R., Foley, J. W., Cartagena, P., Gennari, M., Dublet, B., Casoneda, R., Linsenmayer, T. F., van der Rest, M., Olsen, B. R., and Gordon, M. K. (1993) J. Biol. Chem. 268, 12177-12184
3. Lunstrum, G. P., Morris, N. P., McGough, A. M., Keese, D. R., and Burgeson, R. E. (1991) J. Cell Biol. 113, 963-969
4. Koch, M., Bernasconi, C., and Chiapetti, M. (1992) Eur. J. Biochem. 207, 847-856
5. Gordon, M. K., Gerecke, D. R., Dublet, B., van der Rest, M., and Olsen, B. R. (1989) J. Biol. Chem. 264, 19772-19778
6. Trueb, R., and Trueb, R. (1992) Eur. J. Biochem. 207, 549-557
7. Watt, S. L., Lunstrum, G. P., McGough, A. M., Keese, D. R., Burgeson, B. E., and Morris, N. P. (1992) J. Biol. Chem. 267, 20093-20099
8. Lunstrum, G. P., McGough, A. M., Marinovich, M. P., Keese, D. R., Morris, N. P., and Burgeson, R. E. (1992) J. Biol. Chem. 267, 20097-20099
9. Keese, D. R., Lunstrum, G. P., Morris, N. P., Stoddard, D. W., and Burgeson, R. E. (1991) J. Cell Biol. 115, 971-978
10. Brown, J. C., Mann, K., Wiedemann, B., and Tinpl, R. (1993) J. Cell Biol. 120, 557-567
11. Guilleberg, D., Tingstrom, A., Thuroson, A. C., Olsson, L., Terracio, L., Borg, T. K., and Rubin, R. (1990) Exp. Cell Res. 186, 254-272
12. Schiro, T. A., Chat, B. M., Roswit, T. W., Kinnier, P. D., Henman, M. B., Eisen, A. Z., and Kupper, T. S. (1991) Cell 67, 403-410
13. Klein, C. E., Dressel, D., Steinmayer, T., Mauch, C., Ecke, B., Krieg, T., Bankert, R. B., and Weber, L. (1991) J. Cell Biol. 118, 1427-1436
14. Heine, J., Ignozzi, R. A., Hemler, M. E., Crouse, C., and Massague, J. (1988) J. Biol. Chem. 264, 380-388
15. Tingstrom, A., Heldin, C.-H., and Ruben, K. (1991) J. Cell Sci. 102, 315-322
16. Staat, W. D., Such, K. P., Zutter, M. M., Adams, S. P., Rodriguez, B. A., and Sanz, E. (1993) J. Biol. Chem. 268, 10095-10101
17. Nishiyama, T., Tominaga, N., Nakajima, K., and Hayashi, T. (1988) Collagen Res. 8, 259-273
18. Nishiyama, T., Tsunenaga, M., Nakayama, Y., Adachi, E., and Hayashi, T. (1989) Matrix 9, 199-209
19. Labarca, C., and Paigen, K. (1980) Annu. Rev. Biochem. 52, 344-352
20. Lunstrum, G. P., Sakai, L. Y., Keese, D. R., Morris, N. P., and Burgeson, R. E. (1993) J. Biol. Chem. 261, 9042-9048
21. Morris, N. P., Keese, D. R., Glanville, R. W., Bentz, H., and Burgeson, R. E. (1986) J. Biol. Chem. 261, 5638-5644
22. Backsinger, H. P., Morris, N. P., Lunstrum, G. P., Keese, D. R., Rosenbaum, L. M., Compton, I. A., and Burgeson, R. E. (1990) J. Biol. Chem. 265, 10095-10101
23. Sakai, L. Y., Keene, D. R., Morris, N. P., and Burgeson, R. E. (1986) J. Biol. Chem. 261, 1577-1586
24. Nishiyama, T., Tsunenaga, M., Nakayama, Y., Yamato, M., Adachi, E., and Hayashi, T. (1993) Matrix 13, 447-455
25. Guidry, C., and Grinnell, F. (1987) J. Cell Biol. 104, 1097-1103
26. Kupper, T. S., and Ferguson, T. A. (1993) FASEB J. 7, 1401-1406
27. Sugneu, S. P., Gordon, M. K., Seyer, J., Dublet, B., van der Rest, M., and Olsen, B. R. (1989) J. Cell Biol. 109, 939-945
28. Lethias, C., Descollonges, Y., Garrone, R., and van der Rest, M. (1993) J. Invest. Dermatol. 101, 95-98
29. Schafer, I. A., Shapiro, A., Kovsh, M., Lang, C., and R. B. Fratianne, R. B. (1989) Exp. Cell Res. 183, 112-125