Role of Fibronectin in Collagen Deposition: Fab' to the Gelatin-binding Domain of Fibronectin Inhibits Both Fibronectin and Collagen Organization in Fibroblast Extracellular Matrix

JOHN A. McDONALD, DIANE G. KELLEY, and THOMAS J. BROEKELMANN
Pulmonary Division, Department of Medicine, Washington University School of Medicine at the Jewish Hospital of St. Louis, St. Louis, Missouri 63110

ABSTRACT   We report the effect of Fab' (anti-60k) to a 60,000 mol wt gelatin binding domain of fibronectin (1981, *J. Biol. Chem.* 256:5583) on diploid fibroblast (IMR-90) extracellular fibronectin and collagen organization. Anti-60k Fab' did not inhibit IMR-90 attachment or proliferation in fibronectin-depleted medium. Fibroblasts cultured with preimmune Fab' deposited a dense extracellular network of fibronectin and collagen detectable by immunofluorescence, while anti-60k Fab' prevented extracellular collagen and fibronectin fibril deposition. Matrix fibronectin and collagen deposition remained decreased in cultures containing anti-60k Fab' until cells became bilayered or more dense, when fibronectin and collagen began to appear in lower cell layers. Anti-60k Fab' added to confluent cultures 24 h before fixation and staining had no effect on matrix fibronectin or collagen, so anti-60k Fab' did not simply block immunostaining. Confluent cultures grown in anti-60k Fab' and labeled for 24 h with [³H]proline incorporated identical amounts of [³H]proline and [³H]hydroxyproline, but [³H]hydroxyproline deposition in the cell layer was significantly decreased by anti-60k Fab' ($P < 0.01$). Extracellular matrix collagen does not appear to form a scaffold for fibronectin deposition, as neither gelatin nor a gelatin-binding fragment of plasma fibronectin inhibited deposition of matrix fibronectin. Our results suggest that interstitial collagens and fibronectin interact to form a fibrillar component of the extracellular matrix, and that fibronectin is required for normal collagen organization and deposition by fibroblasts in vitro. Domain-specific antibodies to fibronectin are powerful tools to study the biological role of fibronectin in extracellular matrix organization and other processes.

Fibronectin (FN) is a major synthetic product of diploid fibroblasts (1) organized in discrete extracellular matrix fibrils also containing procollagen types I and III (3, 5, 11, 14, 42). FN is associated with collagen or collagen precursors in extracellular matrix in vivo in human lung specimens (32), in implanted cellulose sponges (26), and during wound healing (13). The distribution of FN and its multiple binding to other matrix components and cells in vitro (36, 39, 45; and references therein) implicate FN in organization of glycosaminoglycans and collagens in extracellular matrix, and in the interaction of cells with these matrix components. However, direct demonstration of FN-mediated organization of other matrix components is lacking.

FN binding to native collagen types I and III in vitro has been demonstrated (8, 18, 20, 24, 35, 40), and FN binds to other native proteins with collagenlike sequences, including acetylcholinesterase (7), and C1q (34). The primary FN-binding site has been localized to the region of the mammalian collagenase cleavage site on collagen (21, 23). However, FN has greater avidity for denatured collagens than for native collagens in vitro, as only native type III collagen binds to FN with affinity similar to that of denatured type I, II, or III collagens (8, 18). The discrepancy between in vitro binding and in situ associations between FN and collagens may be explained by their simultaneous secretion in propinquity (29), indirect binding via glycosaminoglycans (14, 19, 20, 39, 40, 45), or altered

binding properties of extracted collagens. Alternatively, FN may not be involved in collagen organization, and the observed codistribution of FN and collagen may only reflect passive binding. For these reasons, studies with fibroblasts present a more physiological model of FN-collagen interaction than those with isolated components (10, 12, 39, 42).

Previously, we demonstrated that the 60,000 mol wt gelatin binding domain (60k fragment) of FN from limited elastase digestion occupies all FN binding sites on gelatin (31), and Fab′ specific to this domain (anti-60k Fab′) inhibit cellular FN-gelatin binding but not FN-mediated cell adhesion (30). We have studied the effect of anti-60k Fab′ upon the organization of FN and collagen in fibroblast cultures, reasoning that blocking FN-collagen binding should alter collagen deposition, if FN is important in this process. Anti-60k Fab′ inhibits both FN and collagen organization and collagen deposition in extracellular matrix, suggesting the deposition of fibrillar, extracellular FN is essential for similar organization of types I and III collagen in vitro.

MATERIALS AND METHODS

Cell Cultures

Human diploid fibroblasts (IMR-90; The Institute for Medical Research, Camden, N. J.) were cultured in 95% air/5% CO_2 with Dulbecco's modified Eagle's medium (DMEM; Basic Cancer Research Center, Washington University Medical School, St. Louis, Mo.) containing 10% fetal bovine serum (K-C Bilogicals, Lenexa, Kans.) depleted of FN (designated basal medium or BM) (30). Cells between passage number (1/3 subcultivation ratio) 10 and 15 were used. Cultures were supplemented with 25 µg/ml sodium ascorbate (Sigma Chemical Co., St. Louis, Mo.) daily. Cell growth was determined as described (38) on IMR-90 cultured in 96-well microculture plates (Linbro no. 76-003-05; Linbro Div., Flow Laboratories, Inc., Hamden, Conn.).

Immunoreagents

Preimmune Fab′ and affinity-purified anti-60k Fab′ were isolated as described (30). Specificity and bilogical activity of the anti-60k Fab′ used has been established (30). For cell culture, Fab′ were dialyzed (4°C) against Ca^{+2}-, Mg^{+2}-free Dulbecco's phosphate-buffered saline (PBS), then against DMEM, and filter sterilized. Rabbit antiserum against pepsin-extracted human skin type I collagen and fetal calf skin procollagen type III (designated anti-type I and anti-proIII) were generously provided by Katheryn Bradley and Dr. Ronald G. Crystal, Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Md. Antiserum specificity was established by absorption with purified collagens and, for anti-type I, by immunoprecipitation.

To detect FN in the presence of anti-60k Fab′ or 60k fragment, anti-intact FN-Fab′ were absorbed on 60k-fragment Sepharose (30). Nonbound Fab′ (anti-FN minus 60k-Fab′) were biotinylated (biotin/Fab′ of 10–20/1) with the N-hydroxysuccinimide ester of [^{14}C]biotin (BoSu) (New England Nuclear, Boston, Mass.) synthesized as described (16). Fab′ (1.0 mg/ml in 0.1 M NaHCO₃, pH 8.0) were incubated (30 min, 25°C) with 0.3 mM BoSu, unreacted BoSu was inactivated with 0.1 M ethanolamine-HCl/pH 8.0, and biotinylated Fab′ isolated by centrifugation through Sephadex G-25 (31). Avidin-rhodamine-600 conjugates were purchased (Vector Laboratories, Burlingame, Calif.).

To visualize collagens in cultures containing anti-60k Fab′, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (catalog no. 65-173-2; Miles Laboratories, Elkhart, Ind.) was rendered specific to Fc by absorption on rabbit preimmune Fab′-Sepharose-Cl-4B. Fc specificity was established by demonstrating typical FN staining with affinity-purified rabbit anti-human intact FN-IgG (30, 43), and no staining with rabbit anti-intact human FN-Fab′, anti-60k Fab′, or anti-FN minus 60k-Fab′.

Immunofluorescence Staining

Fibroblasts were cultured in Lab-Tek eight-chamber tissue culture slides[1] (Lab-Tek Division of Miles Laboratoires, Inc., Naperville, Ill.) in BM plus the indicated additions. For immunostaining, cells were rinsed with PBS and fixed

---

[1] Washing these slides before use with 1 N HCl/70% ethanol followed by ethanol and PBS rinsing decreases cell losses during processing.

(30 min, 4°C) with periodate-lysine-paraformaldehyde (33). Subsequent steps were carried out for 30 min at room temperature in 7.5 mM Tris-HCl/150 mM NaCl/0.02% NaN₃, pH 7.4, containing 1 mg/ml of heat-denatured (60°C, 15 min) bovine serum albumin (TBS-BSA). For visualization of both FN and collagen in the same specimen, slides were incubated with collagen antiserum (1/30 dilution for anti-type I, 1/50 for anti-proIII), followed by fluoresceinated, Fc-specific goat anti-rabbit IgG (1/30 dilution), biotinylated-anti-FN minus 60k-Fab′ (25 µg/ml), and avidin-rhodamine (25 µg/ml). Wells were rinsed four times with TBS-BSA between changes of immunoreagents. In cultures with anti-60k Fab′, normal rabbit serum controls were run to ensure Fc specificity of the fluoresceinated goat anti-rabbit IgG. Stained slides were mounted in glycerol-PBS (1/9) and examined with an Olympus BH microscope with epifluorescence optics and HBO-100 mercury burner. To avoid bias, similar fields were selected by phase contrast, and fluorescence micrographs were taken.

FITC fluorescence was visualized using an FITC excitation filter, dichroic mirror "B," and a 515-nm barrier filter, and rhodamine with BG-12 and IF-545 excitation filters, dichroic mirror "G," and a 610-nm barrier filter. The rhodamine filter system blocked visualization of FITC labeling completely, but, when intense rhodamine staining was present, this was seen faintly with the FITC filter. Accordingly, in experiments employing anti-60k Fab′ we stained parallel microslide chambers for either FN or collagen. Fluorescence photographs were taken with a UV-FL 40 × objective, NA 0.85, and photoeyepiece NFK 3.3 or 2.5 × with an Olympus PM-10 camera and Kodak Tri-X film exposed for 30 s (rhodamine) or 60 s (FITC) developed with Acufine (Acufine, Inc., Chicago, Ill.) at ~1,200 ASA.

To establish antiserum specificity, antiserum (1/30 dilution for anti-type I, 1/50 for anti-proIII) was incubated with purified collagens (10–20 µg/mL), FN (100 µg/mL), or buffer alone for 1 h at 37°C and overnight at 8°C, centrifuged at 10,000 g for 10 min, and the supernate was used for staining.

Isolation of Procollagens and Collagen for Absorption of Antisera

Confluent IMR-90 were cultured with 20% human serum containing 50 µg/ml of β-aminopropionitrile (BAPN) and ascorbate and [^{14}C]proline (5 µCi/ml, 260 µCi/µmol) for 24 h. This labeled medium was combined with the spent medium (2 liter) of IMR-90 cultured in ascorbate-supplemented BM, and procollagen type I isolated as described (2). Fetal calf skin procollagen type III was isolated by published methods (4). Human skin type I collagen was purified as described (9), and neutral salt-extracted guinea pig skin type I collagen and human placenta type III collagen were provided by Dr. John Jeffrey, Washington University Medical School. Identity of collagens and procollagens was verified by purified Clostridial collagenase digestion and, for procollagens, by pepsin digestion, followed by SDS PAGE with or without reduction (2, 27).

Metabolic Labeling with [^{3}H]Proline and Determination of [^{3}H]Hydroxyproline Synthesis

Cells cultured in 96-well plates in BM or BM plus 100 µg/ml Fab′ for 2 wk were labeled (in triplicate for each condition) by changing to 0.2 ml of fresh BM containing 100 µCi of [^{3}H]proline/ml (130 mCi/µmol; Amersham, Arlington Heights, Ill.) with or without 100 µg/ml Fab′ and culturing for 24 h. After labeling, the medium was removed, the cell layer was rinsed once with PBS, and then the cell layer was removed from the well by adding 0.1 ml of 8 M urea in PBS/1 mM phenylmethylsulfonyl fluoride/25 mM EDTA/5 mM N-ethyl maleimide, and aspirated repeatedly through a 200µl pipette, removed, and combined with a a 0.1-ml rinse of the well with the same solution. Proteinase inhibitors were added to the medium, and both fractions were heated (100°C, 5 min) to inactivate residual proteinases. Medium and cell-layer fractions were dialyzed at 4°C against three 1-liter changes of 0.5 M acetic acid for 48 h at 4°C to remove free [^{3}H]proline, lyophilized, hydrolyzed in constantly boiling HCl for 18 h at 105°C and dried in vacuo. [^{3}H]Proline and [^{3}H]hydroxyproline were determined by amino acid analysis (119C amino acid analyzer, Beckman Instruments, Inc., Palo Alto, Calif.) and liquid scintillation counting. There were no radiolabeled contaminants eluting in the [^{3}H]hydroxyproline region after dialysis of the [^{3}H]proline used for labeling.

Immunoprecipitation

Immunoprecipitation of [^{14}C]proline-labeled fibroblast cell layer extracts was carried out with IgSORB (The Enzyme Center, Boston, Mass.) to precipitate antibody bound antigens as described (43). Immunoprecipitates were displayed by SDS PAGE and fluorography (27, 28).

RESULTS

Effect of Fab' upon Fibroblast Growth and Morphology

IMR-90 plated in BM containing 100 μg/ml of anti-60k Fab' attached and spread normally on glass or tissue culture plastic substrates. There was no difference $(P > 0.1)$ in cell growth between BM, BM plus preimmune Fab', or BM plus anti-60k Fab' (Table I). We also found no difference in incorporation of [^{3}H]thymidine by subconfluent IMR-90 cultured in BM or BM containing 100 μg/ml of anti-60k Fab'. Anti-60k Fab' had no effect upon the normal parallel alignment of IMR-90 (below).

Specificity of Anticollagen Antisera

Fig. 1 displays an immunoprecipitate of IMR-90 cell-layer proteins with anti-type I serum. Two major peptides of 180,000 and 160,000 mol wt comigrating with purified procollagen type I were precipitated from cell-layer extract, along with two smaller peptides which comigrated with pepsin-treated procollagen type I. All precipitated peptides were sensitive to highly purified bacterial collagenase (not shown). Since IMR-90 synthesizes much less type III procollagen than type I, and since the staining pattern obtained with both anticollagen sera was similar, we did not pursue immunoprecipitation with anti-proIII serum.

Staining of confluent IMR-90 cell layers by anti-type proIII or anti-type I serum gave results similar to those of previous investigators (3, 14, 29, 42), revealing an extensive extracellular fibrillar network of collagen (Fig. 2 C-F). Immunostaining by anti-proIII was blocked by purified procollagen type III, but not by type III, I, or FN. Anti-type I staining was blocked by both procollagen I and type I, but not by type III, procollagen type III, or FN (not shown). Thus, the collagen antisera recognized collagenous determinants in IMR-90 extracellular matrix and not FN. However, we could not distinguish between procollagen and processed procollagen by our immunofluorescence methods.

Specificity of our anti-intact FN-IgG, anti-FN minus 60k-Fab', and anti-60k Fab' has been established (30, 43). In this study, methodologic specificity of FN staining by the avidin-biotin system (15) was demonstrated by (a) blocking biotinylated anti-FN minus 60k-Fab' staining with purified FN, (b) blocking staining with avidin-rhodamine by the inclusion of excess biotin or avidin, and (c) showing no staining with biotinylated preimmune Fab' followed by avidin-rhodamine. Neither avidin-rhodamine nor FITC-labeled second antibody
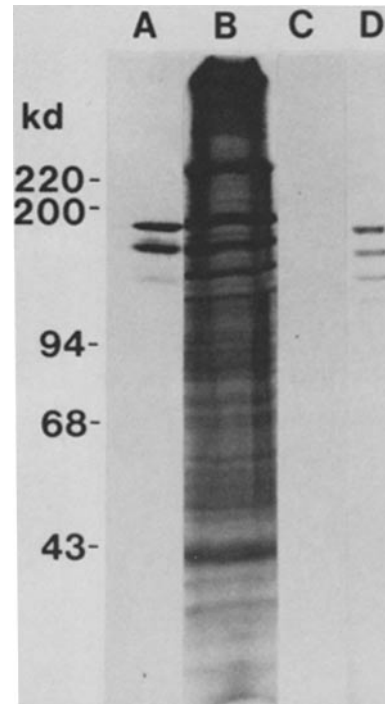

FIGURE 1 Immunoprecipitation of [^{14}C]proline-labeled IMR-90 fibroblast cell layer extract with anti-type I collagen serum. Samples were run on SDS 7.5% PAGE, and labeled polypeptides were displayed by fluorography. Slot A, purified IMR-90 procollagen type I; B, total cell layer extract; C, B immunoprecipitated with preimmune rabbit serum; D, B immunoprecipitated with anti-type I collagen serum. Migration of marker proteins (in kdaltons) is indicated. Note that no labeled bands were precipitated with preimmune serum, and that anti-type I collagen serum precipitated two major polypeptides comigrating with purified procollagen type I, and two minor bands (which comigrated with pepsin-treated procollagen type I).

alone resulted in significant staining. In confluent control cultures stained for both FN and either type I or type III collagen, there was extensive codistribution of both extracellular antigens, as previously described by others (3, 14, 29, 42).

Effect of Fab' upon FN and Collagen Organization in Extracellular Matrix

IMR-90 cultured in FN-depleted medium deposited both FN and collagen types I and III as a complex network of discrete, interconnecting fibrils in extracellular matrix. However, the time-course of FN and collagen appearance differed. FN first appeared beneath isolated cells in linear subcellular strands, and then as short extracellular fibrils associated with elongated cell processes, especially at sites of cell-cell contact (Fig. 2A and B). Collagen in subconfluent cultures appeared in similar extracellular fibers with both anti-type I and anti-pro III sera (Fig. 2 C-F). When double-staining was performed on subconfluent cultures, fibrils with positive collagen staining always stained for FN, but not all FN fibers stained for collagen, as previously described (29).

By marked contrast, fibroblasts cultured with anti-60k Fab' had very little detectable fibrillar extracellular FN, although linear staining for FN was often seen apparently localized beneath cells (Fig. 2 G and H). Subconfluent cells cultured with anti-60k Fab' had virtually no detectable extracellular fibrillar staining with either anti-type I or anti-proIII sera (Fig. 2 I-L).

TABLE I

Effect of Anti-60k Fab' on IMR-90 Growth

Medium	Cells per well,*‡ × 10⁻³, at			
	0 h	23 h	97 h	143 h
Basal	26.2	26.4 ± 2.9	99.6 ± 8.3	123.4 ± 1.7
Basal plus preimmune Fab'	—	28.0 ± 2.5	99.0 ± 8.0	126.5 ± 2.1
Basal plus anti-60k Fab'	—	29.3 ± 2.8	85.4 ± 7.8	131.3 ± 5.2

* Mean ± standard deviation of triplicate wells.
‡ Cell number in different medium not significantly different at any time point by one-way analysis of variance.
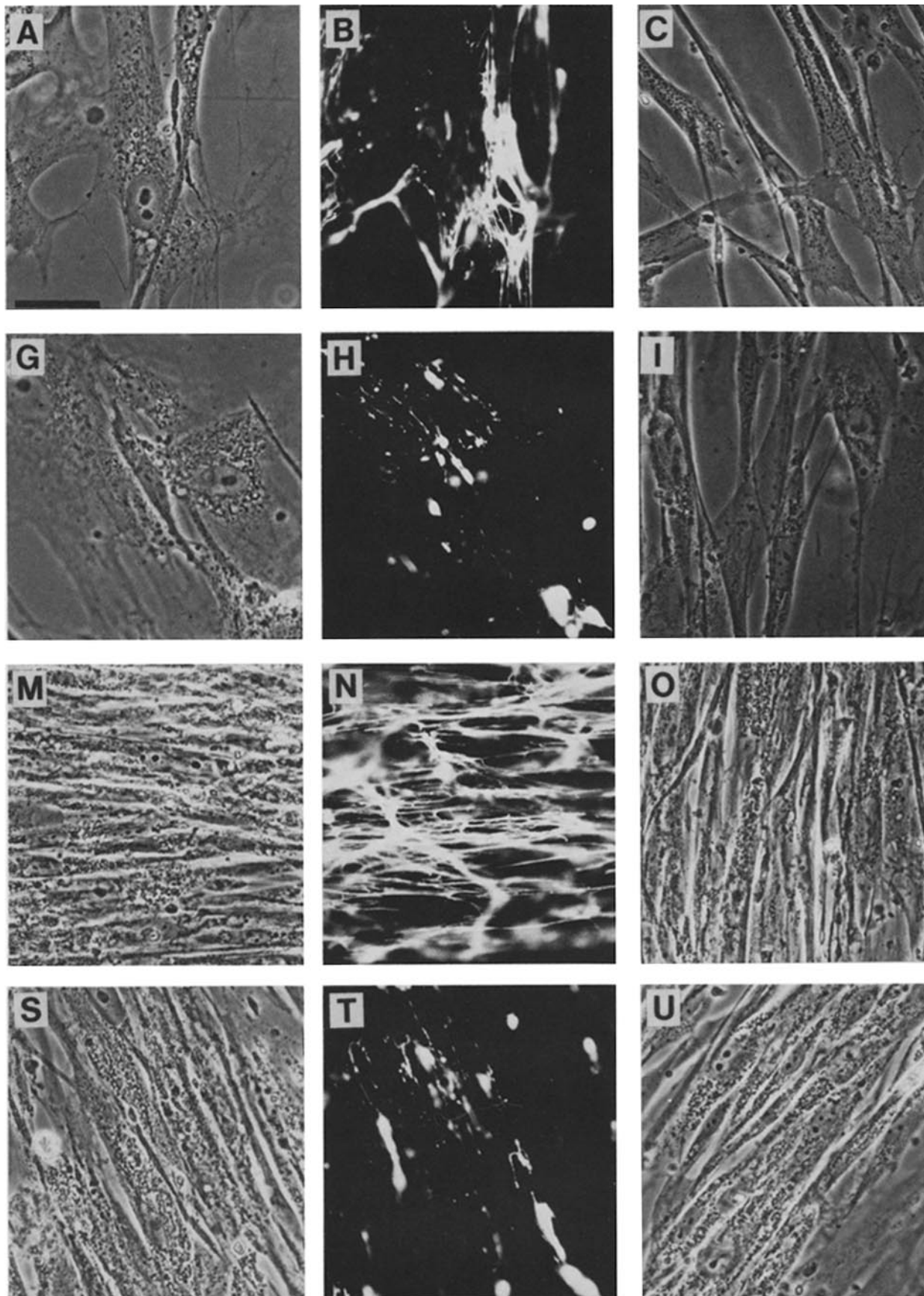
FIGURE 2 Effect of anti-60k Fab' upon FN and collagen in IMR-90 fibroblast extracellular matrix. IMR-90 fibroblasts were cultured in ascorbate-supplemented basal medium containing 100 μg/ml of preimmune Fab' or anti-60k Fab' and stained for FN and collagens at 2 d (A-L) and 7 d (M-X) after plating. The same field is shown by phase and fluorescence microscopy. The top row (A-F) shows cells cultured with preimmune Fab' for 2 d and stained for FN (A and B), type I collagen (C and D), and procollagen type III (E and F). The next row (G-L) shows cells cultured with anti-60k Fab' for 2 d and stained for FN (G and H), type I collagen
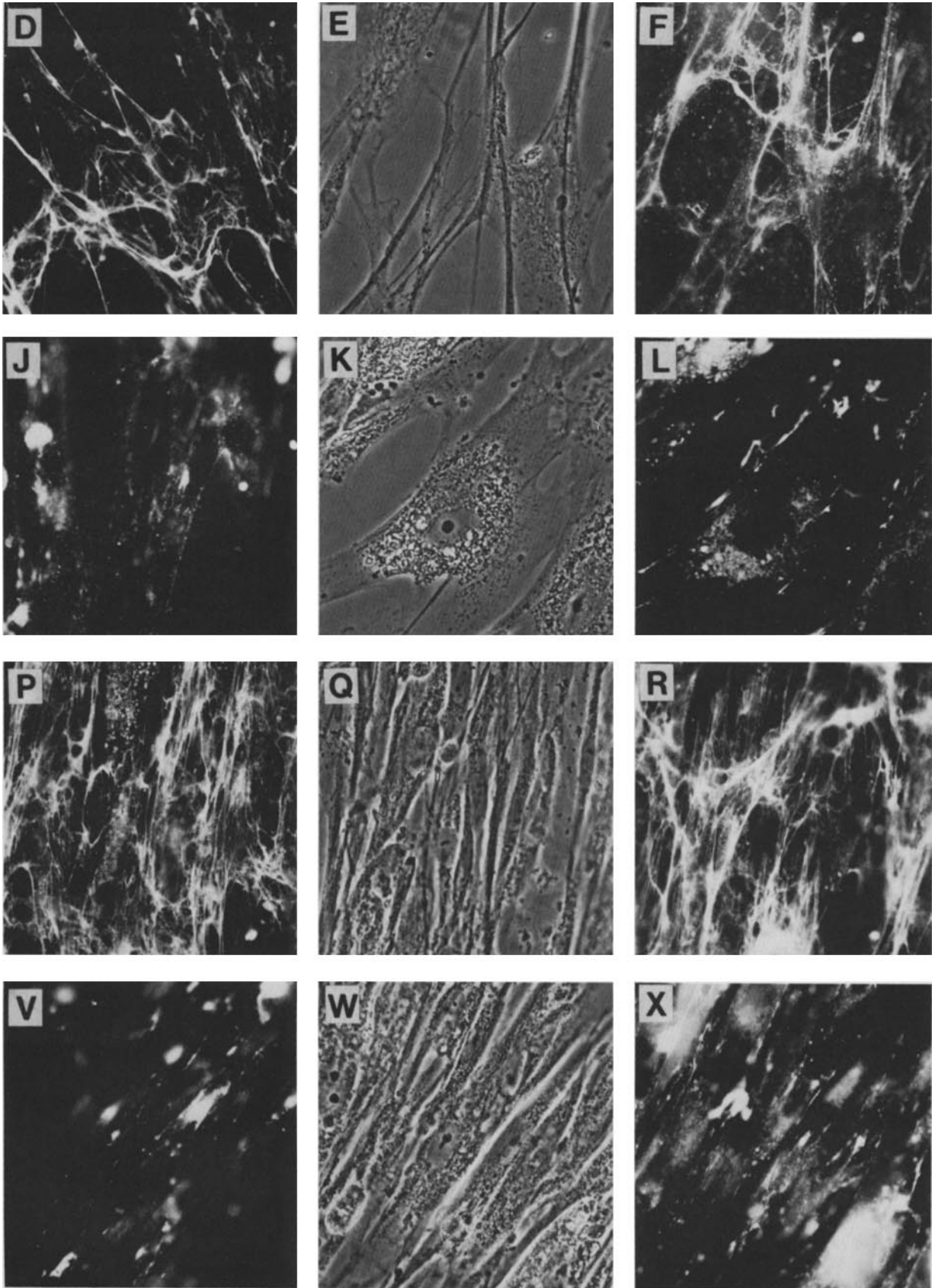
(*I* and *J*), and procollagen type III (*K* and *L*). Note the virtual absence of fibrillar extracellular FN and collagen staining. Row *M–R* demonstrates extensive fibrillar extracellular staining for FN (*M* and *N*), type I collagen (*O* and *P*) and procollagen type III (*Q* and *R*) in confluent (7 d) cells cultured with preimmune Fab'. Row *S–X* shows cells cultured for the same period with anti-60k Fab' and stained for FN (*S* and *T*), type I collagen (*U* and *V*), and procollagen type III (*W* and *X*). Note the marked reduction in extracellular fibrillar FN and collagens, and amorphous deposits of both antigens. The granular perinuclear staining seen especially well in *L*, *P*, and *X* is autofluorescence which was orange colored and not due to FITC. Bar, 50 μm.

As cells cultured in BM approached confluence, an extensive network of extracellular FN and collagen fibrils appeared (Fig. 2 M-R). However, fibroblasts cultured with anti-60k Fab' had almost no detectable fibrillar extracellular FN, while amorphous deposits were seen (Fig. 2 S and T). Confluent cells cultured with anti-60k Fab' had almost no fibrillar extracellular staining (Fig. 2 U-X). After dense confluence was reached (10 d to 2 wk), positive FN and collagen staining appeared in areas of very high cell density in anti-60k Fab'-treated cultures. This staining was usually restricted to the lower layer of cells. However, even under these conditions, collagen deposition over a 24-h period was decreased by biochemical criteria (below).

To exclude the possibility that anti-60k Fab' was simply blocking immunostaining of FN or collagens, we grew cells to confluence in BM, changed the medium to BM plus 100 µg/mL of anti-60k Fab', cultured the cells for an additional 24 h, and stained them for FN and collagen. There was no effect upon either FN or collagen staining, compared with control cells maintained in BM. In addition, by radioimmunoassay, we have shown that binding of anti-FN minus 60k-IgG to FN is not affected by excess anti-60k Fab' (our unpublished observations).

Effect of Gelatin and 60k Fragment upon FN Organization in Extracellular Matrix

Prevention of FN fibril formation by anti-60k Fab' could reflect a requirement for FN binding to a prexisting collagenous matrix. To test this hypothesis, we attempted to inhibit FN-collagen binding by two other mechanisms. First, we attempted to occupy FN-binding sites on collagen with 60k fragment (31) and, second, we tried to occupy the collagen binding site on FN with excess gelatin. Neither 60k fragment (100 µg/ml) nor heat-denatured guinea pig skin collagen (100 µg/ml) affected FN fibril formation (Fig. 3). Interestingly, 60k fragment (100 µg/ml) also had no apparent effect upon collagen distribution as judged by immunofluorescence, although biochemical analysis of collagen deposition under these conditions has not been completed.

Effect of Anti-60k Fab' upon Deposition of [³H]Hydroxyproline in Fibroblast Cell Layer

Neither preimmune nor anti-60k Fab' affected total [³H]-proline and [³H]hydroxyproline incorporation by IMR-90 (Table II). However, anti-60k Fab' significantly inhibited hydrox-
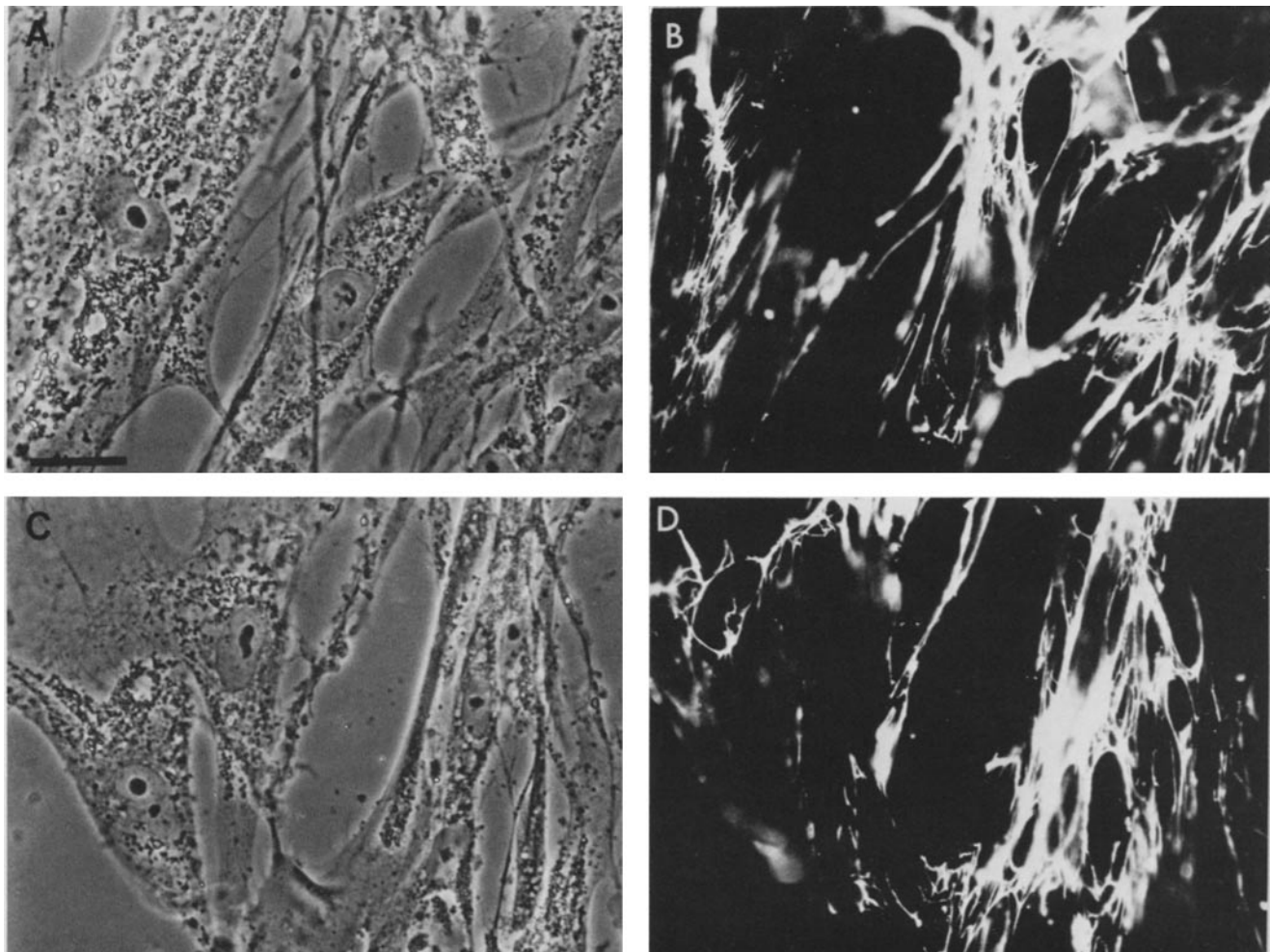

FIGURE 3 Effect of 60k fragment and gelatin upon IMR-90 fibroblast extracellular FN. IMR-90 were cultured for 2 d in BM containing 100 µg/ml of gelatin (A and B) or pure 60k gelatin binding fragment of FN (C and D) and stained for FN. Note the extensive extracellular deposits of fibrillar FN in cells cultured under either condition, indistinguishable from control cultures in basal medium alone (see Fig. 2 A and B). Bar, 50 µm.

TABLE II

Effect of Anti-60k Fab' on [³H]Proline and [³H]Hydroxyproline Content of IMR-90 Medium and Cell Layer

Medium	[³H]cpm,* × 10⁻⁴, in:						
	Cell		Medium		Total proline incorporated‡	Total hypro incorporated‡	Ratio of [³H]-hypro in cell to medium
	Proline	Hypro	Proline	Hypro			
Basal	31.7 ± 1.9	1.18 ± 0.13	12.0 ± 0.6	2.78 ± 0.17	43.7 ± 0.49	3.96 ± 0.28	0.42 ± 0.01
Basal plus preimmune Fab'	29.8 ± 1.5	0.97 ± 0.11	10.4 ± 2.5	2.38 ± 0.48	40.2 ± 1.9	3.35 ± 0.59	0.41 ± 0.04
Basal plus anti-60k Fab'	25.7 ± 2.9	0.74 ± 0.05	12.9 ± 1.7	3.0 ± 0.40	38.6 ± 3.3	3.74 ± 0.36	0.25 ± 0.04§

* All values are ± standard deviation of triplicate cell cultures.
‡ $P > 0.1$ for total [³H]proline and [³H]hydroxyproline content between different media by one-way analysis of variance.
§ Ratio of [³H]hydroxyproline in cell/medium for anti-60k vs. basal or vs. basal plus preimmune $P < 0.01$ by t test.

yproline incorporation in the cell layer, as reflected by the decreased ratio of hydroxyproline in cell layer to medium (Table II). Quantitatively similar results were found in IMR-90 cultured for 1 wk in the presence of anti-60k Fab'. Since collagen hydroxylation was not affected by anti-60k Fab' (based upon identical total hydroxyproline synthesis), anti-60k Fab' inhibited collagen deposition in the extracellular matrix but not total collagenous and non-collagenous protein synthesis.

By immunofluorescence, collagen deposition in cell layer might be expected to decrease much more that was reflected by the decrease in [³H]hydroxyproline content. The large amorphous deposits staining for collagen seen in anti-60k cultures may contain most of the biochemically detectable cell-layer collagen; we may not be visualizing all collagen in the matrix by immunofluorescence, or a significant pool of intracellular [³H]hydroxyproline may have been present during the continuous labeling experiment.

DISCUSSION

Fab' specific to the gelatin-binding domain of FN disrupt both FN and collagen organization in fibroblast extracellular matrix. Neither staining artifacts nor differences in cell density were responsible for this result. While we could not distinguish between procollagen and processed native collagen with the antisera used, collagen (and not FN) was visualized by the collagen antisera. Moreover, metabolic labeling studies confirmed decreased collagen deposition in cell layers in anti-60k Fab'-containing cultures, even in densely confluent cell layers where FN and collagen fibers began to appear. Taken together, these results demonstrate that both organization and deposition of collagen in the extracellular matrix are disrupted by Fab' directed against the gelatin-binding domain of FN.

Similar alterations in fibroblast FN by anti-FN antibody have been reported previously. After 1 h at 37°C, anti-intact FN IgG, but not Fab fragments, caused loss of FN fibrils in human embryonic lung fibroblasts (25). In contrast to our findings that anti-60k Fab' does not affect matrix FN distribution once established, anti-FN IgG also caused partial redistribution of FN fibrils in confluent cultures. Similar results were obtained in primary chick fibroblast cultures, although anti-whole FN Fab as well as IgG induced loss of FN fibrils accompanied by partial retraction of cells from substratum (44). Neither study evaluated the effects of anti-FN IgG or Fab upon collagen organization. Like Yamada (44), we have noted inhibition of IMR-90 attachment and spreading by both anti-intact FN Fab' and domain-specific Fab' to a cell adhesive

fragment of FN (31), emphasizing the utility of anti-60k Fab' for altering FN fibril formation but not cell-FN interaction.

The most likely mechanisms explaining the effects of anti-60k Fab' on both FN and collagen organization are inhibition of FN-collagen binding as well as steric inhibition of FN fibril formation. The latter mechanism is suggested by the recent implication of the gelatin-binding domain of FN as an FN-FN interactive site (6). We have not demonstrated that anti-60k Fab' inhibits FN-native collagen or procollagen binding, even though it inhibits gelatin-cellular FN binding (30). Anti-60k Fab' may inhibit other FN-macromolecule interactions, such as glycosaminoglycan binding, potentially important in collagen deposition (19, 20, 24, 45).

We do not think that a collagen scaffold is necessary for FN fibril formation, as this hypothesis would not explain the concomitant inhibition of collagen organization by anti-60k Fab'. Moreover, ascorbate-deficient primary chick embryo fibroblasts (5) and Chinese hamster overy K-1 cells (our unpublished observations) deposit extracellular FN fibrils, but almost no detectable extracellular collagen. FN deposition in extracellular matrix appears to precede collagen deposition in some fibroblast strains, inasmuch as many FN fibers do not stain for collagen, while fibers staining for collagen usually stain for FN (this study and reference 29). Additionally, neither gelatin nor the purified gelatin-binding domain of FN altered FN organization, as would be expected if native collagens and gelatin bind to the same site or sites on FN, and FN fibril formation required a collagen scaffold.

The relevance of our observations to the participation of FN in collagen fibrilogenesis in vivo remains to be established. Failure of the 60k fragment to inhibit collagen organization could argue against a role of FN in this process. However, if the gelatin-binding domain contains both collagen and FN interactive sites as proposed (6), 60k fragment could even potentiate rather than inhibit collagen-FN codistribution. The relationship between fibroblast matrix fibrils containing FN and collagens (10-12, 41) and native collagen in tissues is not clear. FN and procollagens have been visualized by immuno-electronmicroscopy in 40-nm extracellular fibrils of ascorbate-supplemented human fibroblasts, but typical mature collagen bundles were not present (11). It is clearly established that native collagen molecules and possibly procollagen are capable of in vitro fibril assembly in the absence of FN (17, 41; and references therein), and additional studies are necesary to establish the role of FN in vivo.

Regardless of the mechanisms involved, inhibition of FN fibril formation without affecting growth or general protein synthesis by anti-60k Fab' demonstrates that this domain-

specific antibody is a powerful tool for investigation of the effect of disrupting FN organization upon distribution of other matrix components, and to probe the biological role of FN.

Our results also suggest that the interaction of FN and collagen may be altered without affecting diploid fibroblast growth, a result which was not predicted by previous studies on cell-collagen interaction and chemical alteration of collagen synthesis (22). The reasons for this apparent discrepancy are not clear, but may include alteration of newly synthesized proteins other than collagen by proline analogues, or the inability of anti-60k Fab' to inhibit deposition of subcellular FN. Since adherence is a major determinant of fibroblast growth (37), subcellular FN may be more important in regulating cell behavior than extracellular matrix fibrillar FN (5).

REFERENCES

1. Baum, B., J. A. McDonald, and R. G. Crystal. 1977. Metabolic fate of the major cell surface protein (fibronectin) of normal human fibroblasts. *Biochem. Biophys. Res. Commun.* 79:8–15.
2. Booth, B. A., L. L. Polak, and J. Uitto. 1980. Collagen synthesis by human skin fibroblasts. I. Optimization of the culture conditions for synthesis of type I and type III procollagens. *Biochim. Biophys. Acta.* 607:145–160.
3. Bornstein, P., and J. F. Ash. 1977. Cell surface associated structural proteins in connective tissue cells. *Proc. Natl. Acad. Sci. U. S. A.* 74:2480–2484.
4. Byers, P. H., K. H. McKenney, J. R. Lichtenstein, and G. R. Martin. 1974. Preparation of type III procollagen and collagen from rat skin. *Biochemistry.* 13:5243–5248.
5. Chen, L. B., A. Murray, R. A. Segal, A. Bushnell, and M. L. Walsh. 1978. Studies on intercellular LETS glycoprotein matrices. *Cell.* 14:377–391.
6. Ehrismann, R., M. Chiquet, and D. C. Turner. 1981. Mode of action of fibronectin in promoting chicken myoblast attachment. *J. Biol. Chem.* 254:4056–4062.
7. Emmerling, M. R., C. O. Johnson, D. F. Mosher, B. H. Lipton, and J. E. Lilien. 1981. Cross-linking and binding of fibronectin with asymmetric acetylcholinesterase. *Biochemistry.* 20: 3242–3247.
8. Engvall, E., E. Ruoslahti, and E. J. Miller. 1978. Affinity of fibronectin to collagens of different genetic types and to fibrinogen. *J. Exp. Med.* 78:1584–1595.
9. Epstein, E. H., Jr., R. D. Scott, E. J. Miller, and K. A. Piez. 1971. Isolation and characterization of the peptides derived from soluble human and baboon skin collagen after cyanogen bromide cleavage. *J. Biol. Chem.* 246:1718–1724.
10. Faris, B., R. Sneider, A. Levine, R. Moscaritolo, L. Salcedo, and C. Franzblau. 1978. Effect of ascorbate on collagen synthesis by lung embryonic fibroblasts. *In Vitro* (Rockville). 14:1022–1027.
11. Furcht, L. T., G. Wendelschafer-Crabb, D. F. Mosher, and J. M. Foidart. 1980. An axial periodic fibrillar arrangement of antigenic determinants for fibronectin and procollagen on ascorbate treated fibroblasts. *J. Supramol. Struct.* 13:15–33.
12. Goldberg, B., and H. Green. 1964. An analysis of collagen secretion by established mouse fibroblast lines. *J. Cell Biol.* 22:227–258.
13. Grinnell, F., R. E. Billingham, and L. Burgess. 1981. Distribution of fibronectin during wound healing *in vivo. J. Invest. Dermatol.* 76:181–189.
14. Hedman, K., M. Kurkinen, K. Alitalo, A. Vaheri, S. Johansson, and M. Höök. 1979. Isolation of the pericellular matrix of human fibroblast cultures. *J. Cell Biol.* 81:83–91.
15. Heggeness, M. H., and J. F. Ash. 1977. Use of the avidin-biotin complex for the localization of actin and myosin with fluorescence microscopy. *J. Cell Biol.* 73:783–788.
16. Heitzmann, H., and F. M. Richards. 1974. Use of the avidin-biotin complex for specific staining of biological membranes in electron microscopy. *Proc. Natl. Acad. Sci. U. S. A.* 71:3537–3541.
17. Helseth, D. L. Jr., and A. Veis. 1981. Collagen self-assembly *in vitro.* Differentiating specific telopeptide-dependent interactions using selective enzyme modification and the addition of free amino telopeptide. *J. Biol. Chem.* 256:7118–7128.
18. Jilek, F., and H. Hörmann. 1978. Cold-insoluble globulin (fibronectin). IV. Affinity to soluble collagen of various types. *Hoppe-Seyler's Z. Physiol. Chem.* 359:247–250.
19. Jilek, F., and H. Hörmann. 1979. Fibronectin (cold-insoluble globulin). VI. Influence of heparin and hyaluronic acid on the binding of native collagen. *Hoppe-Seyler's Z. Physiol. Chem.* 360:597–603.
20. Johansson, S., and M. Höök. 1980. Heparin enhances the binding of fibronectin to collagen. *Biochem. J.* 187:521–524.
21. Kleinman, H. K., E. B. McGoodwin, and R. J. Klebe. 1976. Localization of the cell attachment region in types I and II collagen. *Biochem. Biophys. Res. Commun.* 72:426–432.
22. Kleinman, H. K., R. J. Klebe, and G. R. Martin. 1981. Role of collagenous matrices in the adhesion and growth of cells. *J. Cell Biol.* 88:473–485.
23. Kleinman, H. K., E. B. McGoodwin, G. R. Martin, R. J. Klebe, P. P. Fietzek, and D. E. Woolley. 1978. Localization of the binding site for cell attachment in the α_1(I) chain of collagen. *J. Biol. Chem.* 253:5642–5646.
24. Kleinman, H. K., C. M. Wilkes, and G. R. Martin. 1981. Interaction of fibronectin with collagen fibers. *Biochemistry.* 20:2325–2330.
25. Kurkinen, M., and A. Vaheri. 1977. Fibronectin matrix: antibody-induced reorganization in human fibroblast cultures. *Cell Biol. Int. Rep.* 1:469–475.
26. Kurkinen, M., A. Vaheri, P. J. Roberts, and S. Stenman. 1980. Sequential appearance of fibronectin and collagen in experimental granulation tissue. *Lab. Invest.* 43:47–51.
27. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 277:680–685.
28. Laskey, R. A., and A. D. Mills. 1975. Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56:335–341.
29. Ledger, P. W., N. Uchida, and M. L. Tanzer. 1980. Immunocytochemical localization of procollagen and fibronectin in human fibroblasts: effects of the monovalent ionophore, monesin. *J. Cell Biol.* 87:663–671.
30. McDonald, J. A., T. J. Broekelmann, D. G. Kelley, and B. Villiger. 1981. Gelatin-binding domain-specific anti-human plasma fibronectin Fab' inhibits fibronectin-mediated gelatin binding but not cell spreading. *J. Biol. Chem.* 256:5583–5587.
31. McDonald, J. A., and D. G. Kelley. 1980. Degradation of fibronectin by human leukocyte elastase: release of biologically active fragments. *J. Biol. Chem.* 255:8848–8858.
32. McDonald, J. A., B. Villiger, T. Broekelmann, C. Kuhn, and C. Torikata. 1981. Human lung fibronectin: ultrastructural localization by immunohistochemistry. *Clin. Res.* 29:449A.
33. McLean, I. W., and P. K. Nakane. 1974. Periodate-lysine-paraformaldehyde fixative. A new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22:1077–1083.
34. Menzel, E. J., J. S. Smollen, L. Liotta, and K. B. M. Reid. 1981. Interaction of fibronectin with Clq and its collagen-like fragment (CLF). FEBS *(Fed. Eur. Biochem. Soc.)* Lett. 129: 188–192.
35. Mosher, D. F. 1980. Fibronectin. *Prog. Hemostasis Thromb.* 5:111–151.
36. Mosher, D. F., P. E. Schad, and H. K. Kleinman. 1979. Cross-linking of fibronectin to collagen by blood coagulation factor XIIIa. *J. Clin. Invest.* 64:781–787.
37. O'Neil, C. H., P. N. Riddle, and P. W. Jordan. 1979. The relation between surface area and anchorage dependence of growth in hamster and mouse fibroblasts. *Cell.* 16:909–918.
38. Patterson, M. K., Jr. 1979. Measurement of growth and viability of cells in culture. *Methods Enzymol.* 58:143–144.
39. Perkins, M. E., T. H. Ji, and R. O. Hynes. 1979. Cross-linking of fibronectin to sulfated proteoglycans at the cell surface. *Cell.* 16:941–952.
40. Ruoslahti, E., and E. Engvall. 1980. Complexing of fibronectin, glycosaminoglycans and collagen. *Biochim. Biophys. Acta.* 631:350–358.
41. Trelstad, R. L., and K. Hayashi. 1979. Tendon collagen fibrillogenesis: intracellular subassemblies and cell surface changes associated with fibril growth. *Dev. Biol.* 71:228–242.
42. Vaheri, A., M. Kurkinen, V.-P. Lehto, E. Linder, and R. Timpl. 1978. Codistribution of pericellular matrix proteins in cultured fibroblasts and loss in transformation: fibronectin and procollagen. *Proc. Natl. Acad. Sci. U. S. A.* 75:4944–4948.
43. Villiger, B., D. G. Kelley, W. Engleman, C. Kuhn III, and J. A. McDonald. 1981. Human alveolar macrophage fibronectin: synthesis, secretion, and ultrastructural localization during gelatin-coated latex particle binding. *J. Cell Biol.* 90:711–720.
44. Yamada, K. M. 1978. Immunologic characterization of a major transformation-sensitive fibroblast cell surface glycoprotein. *J. Cell Biol.* 78:520–541.
45. Yamada, K. M., D. W. Kennedy, K. Kimata, and R. M. Pratt. 1980. Characterization of fibronectin interactions with glycosaminoglycans and identification of active proteolytic fragments. *J. Biol. Chem.* 256:6055–6063.