In Vitro Cell. Dev. Biol.—Animal 34:203–210, March 1998

1071-2690/98 $05.00 + 0.00

FIBRONECTIN PRODUCTION BY CULTURED HUMAN LUNG FIBROBLASTS IN THREE-DIMENSIONAL COLLAGEN GEL CULTURE

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(Received 17 February 1997; accepted 10 May 1997)

SUMMARY

In vivo, fibroblasts are distributed in a three-dimensional (3-D) connective tissue matrix. Fibronectin is a major product of fibroblasts in routine cell culture and is thought to regulate many aspects of fibroblast biology. In this context, we sought to determine if the interaction of fibroblasts with a 3-D matrix might affect fibronectin production. To examine this hypothesis, fibronectin production by fibroblasts cultured in a 3-D collagen gel or on plastic dishes was measured by ELISA. Fibroblasts in 3-D gel culture produced more fibronectin than those in monolayer culture. Fibroblasts in 3-D culture produced increasing amounts of fibronectin when the collagen concentration of the gel was increased. The 3-D nature of the matrix appeared to be crucial because plating the fibroblasts on the surface of a plastic dish underneath a collagen gel was not different from plating them on a plastic dish in the absence of collagen. In addition to increased fibronectin production, the distribution of the fibronectin produced in 3-D culture was different from that of monolayer culture. In monolayer culture, more than half of the fibronectin was released into the culture medium. In 3-D culture, however, approximately two-thirds remained in the collagen gel. In summary, the presence of a 3-D collagen matrix increases fibroblast fibronectin production and results in greater retention of fibronectin in the vicinity of the producing cells.

Key words: fibronectin, collagen, matrix, fibroblast, 3-dimensional.

INTRODUCTION

In vivo, fibroblasts are distributed in a three-dimensional (3-D) extracellular matrix. Fibroblasts both produce components of this matrix and interact with it. Fibronectin is a multifunctional extracellular matrix glycoprotein that participates in cell adhesion (1), migration (30), phagocytosis (9), and extracellular matrix formation (14), and is a product of fibroblasts (4). As a result, fibronectin is thought to play a major role both in fibroblast matrix production and in the ability of cells to interact with extracellular matrix. In this regard, we sought to determine if the interaction of fibroblasts with a 3-D matrix might alter fibronectin production.

To investigate this question, we first developed a method to solubilize a collagen gel with collagenase in a manner which would permit accurate quantification of fibronectin by immunoassay. We then determined the flux of known concentrations of fibronectin from collagen gels. We then examined the fibronectin production of fibroblasts cultured in a 3-D collagen gel and compared this to monolayer culture. We thus determined that fibroblasts produced more fibronectin in a 3-D collagen gel than in monolayer culture. Furthermore, much of the fibronectin produced by fibroblasts in a 3-D collagen gel culture is transiently retained in the gel and then gradually released into the culture medium.

MATERIALS AND METHODS

Cell and culture conditions. Normal human fetal lung fibroblasts, HFL-1, were purchased from American Type Cell Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM: GIBCO, Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS; Biofluids, Rockville, MD), containing 50 U of penicillin per ml, 50 μg of streptomycin (GIBCO) per ml, and 2 μg of amphotericin B (GIBCO) per ml at 37° C in 5% CO_2. Cultures were routinely passaged after trypsinization every 3–5 d.

Human fibronectin enzyme-linked immunosorbent assay (ELISA). An indirect inhibition ELISA for human fibronectin was used to quantify fibronectin (24). Human fibronectin was purified from human serum by the technique as previously reported (10). It was confirmed (data not shown) that fetal calf serum fibronectin was not detected with the antihuman fibronectin antibody used in the assay, as has been previously reported (25).

Preparation of collagen gels. 3-D collagen gels were prepared essentially as described (31). In brief, we extracted type I collagen by stirring adult rat tail tendons for 48 h at 4° C in sterile 4 m*M* acetic acid. Reconstitution of collagen fibers was achieved by quickly mixing the resulting collagen stock solution with 4× DMEM and FCS. Final concentration of collagen in the gel was adjusted to 0.5 mg/ml. FCS was supplemented to 10%, except as noted in dose-response studies. Two ml of solution was pipetted into a 35-mm culture dish, and gel formation occurred in about 5 min at 37° C.

Standard curve of fibronectin extracted from the collagen gel. Purified fibronectin was mixed with 0.5 mg/ml of collagen solution in the presence of DMEM with 10% FCS. Final concentrations of the fibronectin were adjusted from 39 to 5000 ng/ml. Two ml of each solution were dispensed into 35-mm dishes (FALCON; Becton Dickinson Labware, Lincoln Park, NJ) and allowed to gel. After a gel formed, 0.5 ml of collagenase (0.25 mg/ml, type XI collagenase from Sigma Chemical Co., St. Louis, MO) was added on top of the gels. The time necessary for complete liquefaction of the gel was usually 2 h at 37° C. Fibronectin levels in the resulting solution were then assayed by ELISA.

The collagenase used in the current study contains a variety of proteolytic activities capable of degrading proteins other than collagen. Although pre-

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vious studies have demonstrated that the antigenic epitopes detected in the ELISA used are relatively resistant to limited proteolysis (24,26), we conducted a preliminary series of experiments designed to determine if the collagenase solution would affect the ELISA. Extended digestion did affect the ELISA, but this effect could be blocked by including at least 1% fetal calf serum during the digestion (data not shown). All studies were conducted in the presence of 10% FCS except as noted below because this is a "traditional" growth medium for fibroblasts both in dish and gel culture. For the serum dose experiments, cultures were supplemented with serum to 1% FCS if needed after culture and immediately before extraction.

Flux of fibronectin from the collagen gel. To evaluate the flux of fibronectin from the inside of the collagen gel into the supernatant, purified fibronectin (final concentration, 2000 ng/ml) was added to a 0.5 mg/ml collagen gel in the presence of DMEM with 10% FCS. The final volume of the resulting gel was 2.0 ml. After gels formed, they were covered with 2 ml DMEM with 10% FCS and incubated for 1, 2, and 3 d. On each day, the supernatants were harvested and the gels were digested by the procedures described above.

To study the effect of collagen concentration on the flux of fibronectin from the gel to the media, we added the same concentrations of fibronectin to gels containing 0.125, 0.25, 0.5, 1.0 mg of collagen per ml in the presence of DMEM with 10% FCS. After a 3-d incubation, the supernatants and the gels were harvested. Fibronectin was assayed by ELISA.

Fibronectin production by fibroblasts in 3-D culture. For 3-D culture, 1×10^5 HFL-1 fibroblasts were mixed in a 0.5 mg/ml of collagen solution in the presence of DMEM with 10% FCS and dispensed into 35-mm dishes. The mixture was then allowed to gel at 37° C. Two ml of media were then added on top of the gels. Two fractions of fibronectin were assayed, media and gel/cell associated. As it was not possible to determine if fibronectin bound to cell surfaces had been released by the digestion, the gel and cell-associated portions were combined. To harvest these samples, after a 24-h incubation of fibroblasts cast into 3-D collagen gels, the supernatant media were changed to fresh media. From replica cultures, media were removed and the gels were rinsed with serum-free DMEM and digested with collagenase to determine the baseline content of fibronectin. The solubilized gels were collected into tubes, any cells remaining on the dishes were scraped into 0.05 *M* Na_3PO_4 with 2 m*M* phenylmethylsulfonyl fluoride (PMSF, Sigma) and 1% Triton X-100 (Sigma), and sonicated after combination with the solubilized gels (27). On Day 2, in replica cultures, media were harvested and saved for assay and the gels and cell layers were harvested as on Day 1.

For monolayer culture, 1×10^5 of the cells were plated in 35-mm dishes in DMEM with 10% FCS. On Day 1, media were changed and cell layers were harvested as described above. On Day 2, in replica cultures, media were harvested for assay, and cell layers were again harvested.

Fibronectin levels were assayed by ELISA. The amount of the cell/gel-associated fibronectin produced from Day 1 to Day 2 was assessed by subtracting the fibronectin contents of Day 1 from those of Day 2 cultures. To determine cell number, we assayed the amount of DNA by measuring fluorescence after staining with Hoechst dye 33258 (16). To avoid influence of variable cell numbers, fibronectin production was normalized as ng fibronectin/mg DNA in all cultures. To accomplish this, replica cultures were treated by the same procedure as that used to quantify fibronectin. After supernatants were removed, gels were washed; then collagenase was added on top of the gels to digest them. The solubilized gel was transferred into a tube, and remaining cells were removed with trypsin. The trypsin solution including cells was combined with the gel solution and centrifuged, and the cell pellet was stored at −80° C until assay. We assayed the amount of DNA in the cell pellet by measuring fluorescence after staining with Hoechst dye 33258 (16). Quantification of cell number by DNA was chosen because this method provided more reliable results than direct enumeration of cell number (18).

Northern blot analysis. To determine whether changes in fibronectin production were accompanied by altered mRNA levels, Northern blot analysis was done. Cells were cultured for either 12 or 24 h in dish culture or embedded in collagen gels as described above. RNA was then extracted by the methods of Chomczynski and Sacchi (8), by guanidinium thiocyanate extraction. Ten micrograms of RNA from each condition was electrophoresed on an 0.8% agarose/formaldehyde gel, and Northern blot transfer to nylon membrane (Nytran; Schleicher & Schuell, Keene, NH) was performed with a rapid downward transfer system (TurboBlotter, Schleicher & Schuell). The blots were hybridized with a ^{32}P-labeled human fibronectin cDNA probe (gift of F. Baralle, Oxford, UK). After autoradiography, the blots were rehybridized with a ^{32}P-labeled glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA.

Effect of collagen concentration, initial cell density and serum concentration on fibronectin production. To test the effect of collagen concentration on fibronectin production in 3-D culture, 1×10^5 HFL-1 fibroblasts were cast in 0.125, 0.25, 0.5, and 1.0 mg/ml collagen solutions in the presence of DMEM with 10% FCS. To examine the effect of initial cell density on fibronectin production, 0.25, 0.5, 1, 2, and 4×10^5 cells were cast in 0.5 mg/ml collagen gels in the presence of 10% FCS. To study the effect of serum concentration on fibronectin production, 1×10^5 cells were cultured in 1, 5, 10, and 20% of FCS. The media were changed on Day 1, and the media, gels, and cell layers were harvested as described above.

Effect of position of fibroblasts and gel contraction on fibronectin production. To study the effect of the position where cells were plated in the collagen gel on fibronectin production, cells were plated in monolayer culture and in the following three different conditions using collagen gels: 1) "In Gel": 3-D culture of 1×10^5 HFL-1 fibroblasts performed as described above; 2) "On Gel": the same number of cells plated on top of an identically constructed collagen gel which did not contain any cells; and 3) "Under Gel": the same number of cells cultured in monolayer culture for 24 h. Following this, media were removed and a collagen gel constructed in an identical manner but containing no additional cells was placed on top of the cell layers. The media, cell layers, and gels were harvested after an additional 24 h from each of the cultures as described above.

To determine if surrounding fibroblasts on all sides with collagen or if the 3-D culture was the major determinant regulating fibroblast fibronectin production, plastic dishes were coated with a thin layer of type I collagen by incubating each dish with 30 μg collagen per ml in distilled water for 15 min (5). Fibroblasts were then plated on the dishes under and in 3-D native collagen gels as described above. Fibronectin production was then determined as described above except that fibronectin produced both in the interval between 24 and 48 h after plating and between 48 and 72 h after plating was assessed.

To evaluate the effect of gel contraction on fibronectin production by the cells in 3-D culture, collagen gels containing 1×10^5 HFL-1 fibroblasts were released from the surface of the culture plate after 1 d of culture. The media were changed, and the gels gently rocked. The floating gels were then allowed to contract (6). The media, cell layers, and gels were harvested after 24 h as described above.

Effect of TGF-β on fibronectin production in 3-D culture. TGF-β has been demonstrated to have a stimulating effect on fibronectin production by fibroblasts in monolayer culture (33). To test the effect of TGF-β on fibronectin production in 3-D culture, 1×10^5 of cells were plated in monolayer or 3-D cultures. On Day 1, the media were changed and 100 p*M* of TGF-β (R&D system, Minneapolis, MN) was added. Replica dishes containing gels and cell layers were harvested to measure the amount of fibronectin present after 1 d of culture. After another d in culture, the media, gels, and cell layers were harvested.

Statistical analysis. Significance was determined by Student's unpaired *t*-test to compare two samples values, and ANOVA to study the effect of collagen concentration in a dose-dependent manner.

Results

Standard curve of fibronectin in collagen gel. Standard fibronectin samples spiked into a collagen gel could be assayed reliably with collagenase digestion followed by ELISA (Fig. 1). Statistical analysis revealed a significant regression ($y = 1.012x + 10.955$, $P < 0.0001$) of the levels of fibronectin measured by ELISA to those added in the collagen gel in the range between 39 and 5000 ng of fibronectin per ml.

Flux of fibronectin from the collagen gel. Fibronectin added to the collagen gel was gradually released from the gel into the supernatant during a 3-d incubation (Fig. 2). Although most of the fibronectin added to the collagen gel remained in the gel after a 24-h incubation (96.6 ± 6.0%), the fibronectin was distributed almost equally on Day 3 (59.5 ± 2.0% in the gel).

The rate of release of fibronectin from the collagen gel to the media was dependent on the concentration of collagen (Fig. 3). Although 77.0 ± 1.1% of the fibronectin added to the collagen gel remained

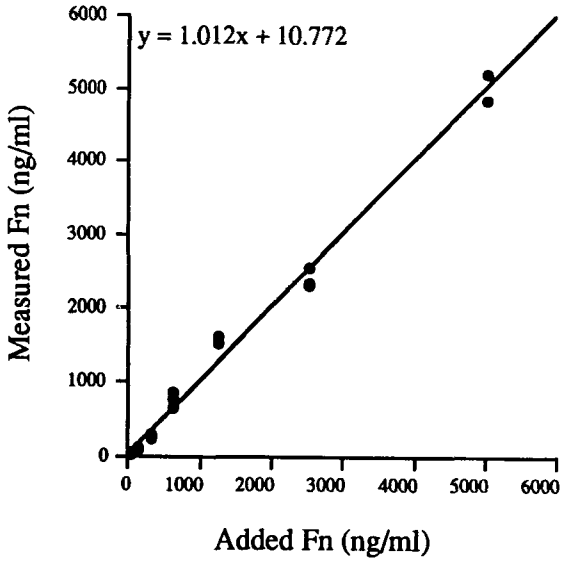

FIG. 1. Standard curve of fibronectin (Fn) assayed in collagen gel. Several concentrations of fibronectin extracted by gelatin-affinity chromatography were added into 0.5 mg/ml collagen gels in the presence of 10% FCS. Fibronectin levels were measured by ELISA after digestion with collagenase. *Vertical axis*: amount of fibronectin measured per gel. Each data point is a measurement of one of triplicate plates used at each concentration. There was a significant regression of measured fibronectin titers to added fibronectin levels ($P < 0.0001$).

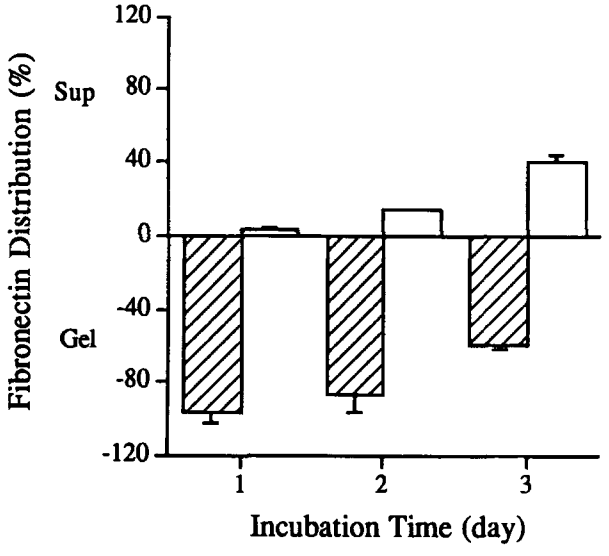

FIG. 2. Flux of fibronectin from collagen gel to supernatant media. Fibronectin extracted by gelatin-affinity chromatography was added into 0.5 mg/ml collagen gels in the presence of 10% FCS, and then incubated for 1, 2, and 3 d. The amount of fibronectin in the media and the gel was measured by ELISA and values were represented as distribution of fibronectin. The fibronectin present in the collagen gels (*hatched bars*) decreased, whereas that in the supernatants (*open bars*) increased with increasing time. Values are means of triplicate plates with *error bars* representing standard error.

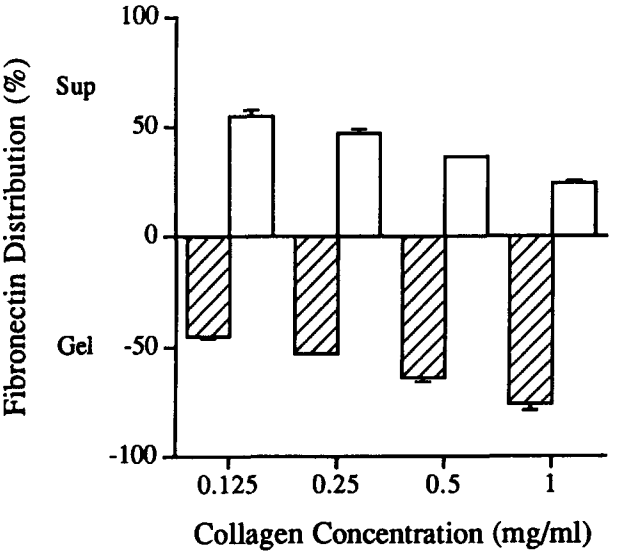

FIG. 3. Flux of fibronectin from different concentration collagen gels to supernatant media. Fibronectin extracted by gelatin-affinity chromatography was added to 0.125, 0.25, 0.5, and 1.0 mg/ml collagen gels in the presence of 10% FCS, and then incubated for 3 d. The amount of fibronectin in the supernatants (*open bars*) and the gels (*hatched bars*) was measured by ELISA. Values represent the distribution of fibronectin. The greater the concentration of collagen used to make the gel, the more fibronectin remained in the gel. Values are means of triplicate plates with *error bars* representing standard error.

in a 1.0 mg/ml of collagen gel after 3 d of incubation, distributions of fibronectin in gels with 0.25 and 0.125 mg/ml of collagen were nearly equal (53.1 ± 1.8, 45.1 ± 2.1% in the gel, respectively).

Fibronectin production by fibroblasts in 3-D culture. There are many reports that proliferation of cells in collagen gels stops after the commencement of gel contraction. In our preliminary study, DNA levels of fibroblasts in monolayer culture became significantly higher than those in 3-D culture after 3 d of culture (data not shown). To minimize the effect of gel contraction, we did not allow the gels to float and cultured the cells in the gels for only 2 d. To avoid the influence of trypsinization and plating on cell behavior, cell/gel-associated fibronectin amounts on Day 1 were subtracted from those on Day 2. Therefore, fibronectin amounts described in our culture studies represent fibronectin production from Day 1 to Day 2.

Total fibronectin production by cells in 3-D culture from Day 1 to Day 2 was significantly higher than in monolayer culture (Fig. 4). Distribution of fibronectin under the two conditions was different. Although more than half of the fibronectin was released into the supernatant media in monolayer culture, approximately two-thirds of the fibronectin was present in gel/cell layer in 3-D culture.

Northern blot analysis. Northern blot analysis of mRNA extracted from fibroblasts cultured under control conditions and in 3-D gels demonstrated no major difference in fibronectin mRNA relative to GAPDH after either 12 or 24 h of culture (Fig. 5).

Effect of collagen concentration, initial cell density and serum concentration on fibronectin production. Increasing the concentration of collagen used to make the gels resulted in increasing fibronectin production in a concentration-dependent manner ($P < 0.05$ by ANOVA, Fig. 6). In 3-D culture with 1 and 0.5 mg/ml of collagen gel,

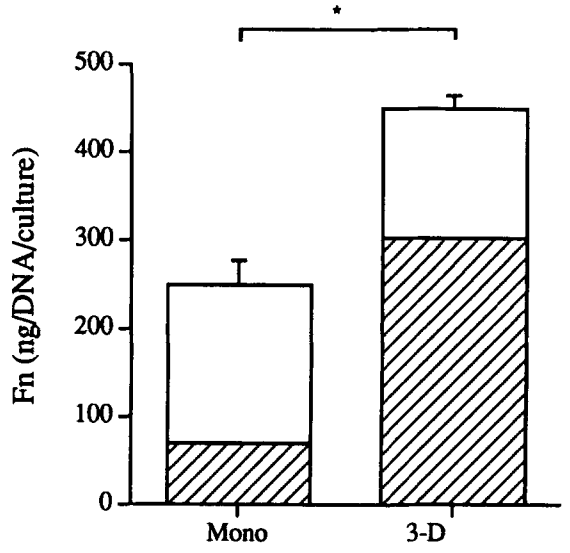

FIG. 4. Quantification of fibronectin in monolayer and 3-D culture. Cells were cultured in monolayer or 3-D culture for 2 d in the presence of 10% FCS. On Day 1, media were changed, and cell layers and gels were harvested. On Day 2, media, cell layers, and gels were harvested. The amounts of fibronectin in all samples were assayed by ELISA. Fibronectin levels in supernatant (*open bars*) represent the amount of fibronectin in supernatants after changing on Day 1 until Day 2. Cell/gel-associated fibronectin (*hatched bars*) levels were calculated by subtracting cell/gel-associated fibronectin on Day 1 from those on Day 2. These fibronectin levels were divided by the amount of DNA measured on Day 2 in both cultures to normalize fibronectin production to cell number. *Vertical axis*: fibronectin released normalized to DNA. *Horizontal axis*: culture condition. Total fibronectin released by cells in 3-D culture from Day 1 to Day 2 was significantly higher than that in monolayer culture (*, $P < 0.01$). The distribution of fibronectin in each condition was also different. Although more than half of the fibronectin was released into supernatant media in the monolayer culture, approximately two-thirds of fibronectin was present in the gel/cell layer in 3-D culture. Values are means of triplicate plates with *error bars* representing standard error.

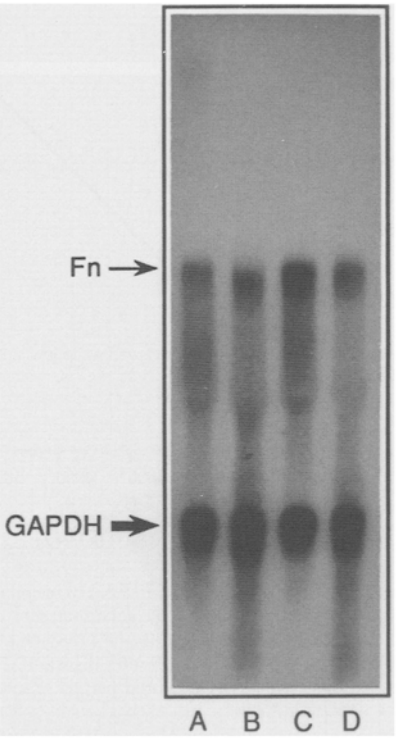

FIG. 5. Autoradiogram of Northern blot. Fibroblasts were cultured under control (dish) culture or embedded in 3-D collagen gels as described in "Materials and Methods." After either 12 or 24 h of culture, mRNA was extracted and Northern blot analysis performed for either fibronectin (*thin arrow*), or for comparison, GAPDH (*thick arrow*). There was no difference in fibronectin mRNA expression relative to GAPDH. *Lane A*: control culture, 12 h; *lane B*: gel culture, 12 h; *lane C*: control culture, 24 h; *lane D*, gel culture, 24 h.

the amounts of fibronectin were significantly higher than in monolayer culture ($P < 0.05$). There was no significant difference between the lowest concentration of collagen tested in 3-D culture and in the monolayer culture.

Initial cell density (0.25, 0.5, 1, 2, and 4×10^5 cells/culture) used to prepare the collagen gels had no effect on fibronectin production in 3-D culture (Fig. 7). Serum, in contrast, affected fibronectin production. The higher the concentration of serum used to make the gels, the more fibronectin was produced by the cells in 3-D culture ($P < 0.01$ by ANOVA, Fig. 8).

Effect of position of fibroblasts on fibronectin production. When cells were plated in, on, or under the collagen gel, fibronectin production differed (Fig. 9). In addition, the distribution of fibronectin was different under the various conditions. In the "standard" 3-D culture condition (In Gel), approximately two-thirds of the fibronectin was present in the gel/cell layer. However, fibronectin was distributed evenly when the cells were plated on top of the collagen gel (On Gel). In contrast, most of the fibronectin from the cells cultured under the collagen gel was present in the gel/cell layer (Under Gel). Total fibronectin production in "In Gel" and "On Gel" conditions was higher than in "Under Gel" and "Monolayer" culture. There was no difference between "Under Gel" and "Monolayer."

To help determine if it was the 3-D structure which altered fibronectin production or if this was a result of the cells being surrounded on all sides by collagen, cells were plated on plastic dishes coated with type I collagen and then cultured in dish culture under a collagen gel and within the collagen gel. There was no difference in fibronectin production between cells cultured on collagen and cultured on plastic (Fig. 10). In contrast, cells cultured on collagen-coated dishes and under a collagen gel produced more fibronectin than cells cultured on plastic and under a collagen gel, although the amount of fibronectin produced was significantly less than that produced when cells were cultured within the gel (Fig. 10). This slight increase in cells cultured on collagen under a collagen gel was a strikingly consistent finding. In six separate experiments, cells cultured on collagen-coated dishes under a collagen gel produced 140 ± 28% of the fibronectin produced by fibroblasts cultured on plastic under a collagen gel. This contrasts with the 113 ± 27% produced on collagen-coated dishes compared to plastic in "routine" culture. Some of the fibroblasts could be observed to migrate from the dish into the gel, particularly in collagen-coated dishes (not shown), suggesting the "under gel" culture might be progressing toward an "in gel" system. In support of this, when the cells cultured under the collagen gel were allowed to incubate for 48 h and then fibronectin production was measured over the subsequent 24 h, no difference could be observed between "under gel" and "in gel" conditions (20.7 ± 1.7 vs 21.6 ± 1.6 ng/mg DNA, $P < 0.2$).

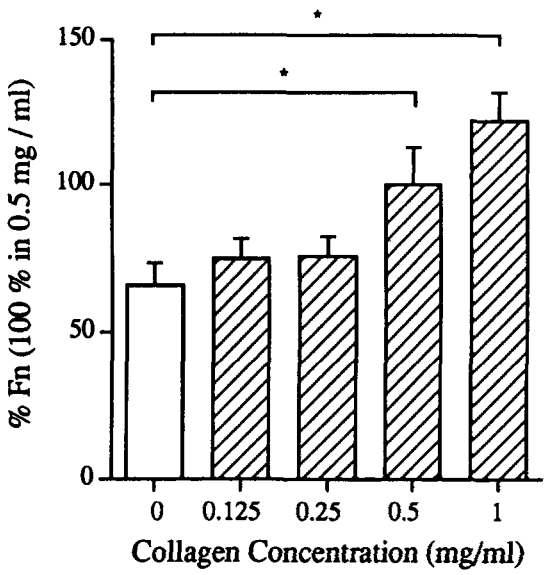

FIG. 6. Effect of collagen concentration on fibronectin levels in 3-D culture. Fibroblasts were cultured in 0.125, 0.25, 0.5, and 1.0 mg/ml collagen gels (*hatched bars*) and in monolayer culture (*open bar*) for 2 d in the presence of 10% FCS. Fibronectin production was calculated by the difference between Day 1 and Day 2 cultures and expressed as ng fibronectin DNA to normalize to cell number as described above. *Vertical axis*: per cell fibronectin production expressed as a percentage of that of cells in a 0.5 mg/ml collagen gels. *Horizontal axis*: collagen gel concentration. The higher the concentration of collagen used to make the gels, the more total fibronectin was produced ($P < 0.05$ by ANOVA). Fibronectin levels in 1 and 0.5 mg/ml of collagen gel were significantly higher than in monolayer culture (*, $P < 0.05$). Fibronectin levels were assayed by ELISA. Values are means of triplicate plates with *error bars* representing standard error.

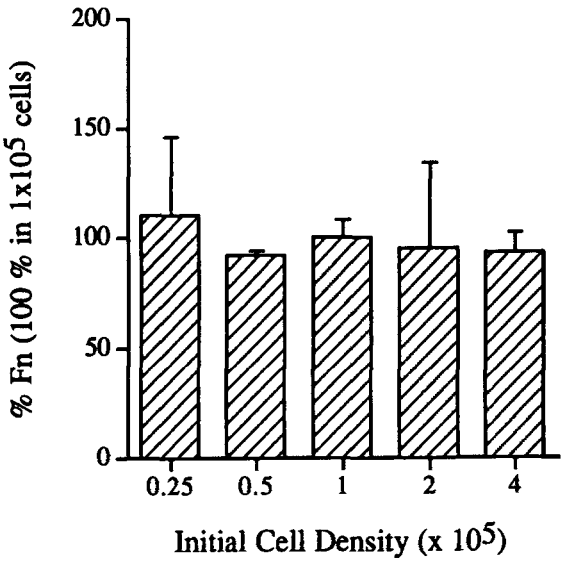

FIG. 7. Effect of initial cell density on fibronectin production by fibroblasts in 3-D culture. Different densities of cells (0.25, 0.5, 1, 2, and 4 × 10^5/culture) were plated in 0.5 mg/ml of collagen gel and cultured for 2 d. Fibronectin production was calculated by the difference between Day 1 and Day 2 cultures and expressed as ng fibronectin DNA in order to normalize to cell number as described above. *Vertical axis*: per cell fibronectin production expressed as a percentage of that of 10^5 plated cells. *Horizontal axis*: number of cells initially plated. There was no significant relationship between the initial cell density and fibronectin production in 3-D culture. Values are means of triplicate plates with *error bars* representing standard error.

Effect of gel contraction on fibronectin production. Fibroblasts cultured in contracted gels produced less fibronectin than in gels which were unable to contract horizontally ($P < 0.01$), and production was not different from the production in monolayer culture (Fig. 11).

Effect of TGF-β on fibronectin production in 3-D culture. The effect of TGF-β on fibronectin production by fibroblasts in 3-D culture was similar to that in monolayer culture. TGF-β stimulated a 1.54 ± 0.12-fold increase in fibronectin production in monolayer culture and a 1.72 ± 0.13-fold increase in 3-D culture (Fig. 12). These stimulations were significant ($P < 0.05$ and 0.01, respectively).

DISCUSSION

The present study demonstrates that fibroblasts cultured in a 3-D collagen gel produce more fibronectin than those in monolayer culture. Fibroblasts in 3-D culture produce increasing amounts of fibronectin when the collagen concentration of the gel is increased, if serum concentrations are increased and in the presence of TGF-β. Increasing the density of fibroblasts in the gel did not affect fibronectin production. Finally, the 3-D nature of the matrix appeared to be crucial because plating fibroblasts on the plastic dish underneath a collagen gel was not different from plating them on a plastic dish in the absence of collagen. Similarly, coating the dish with a thin air-dried collagen layer did not increase fibronectin production. Thus, the presence of a 3-D collagen matrix can alter fibroblast fibronectin production.

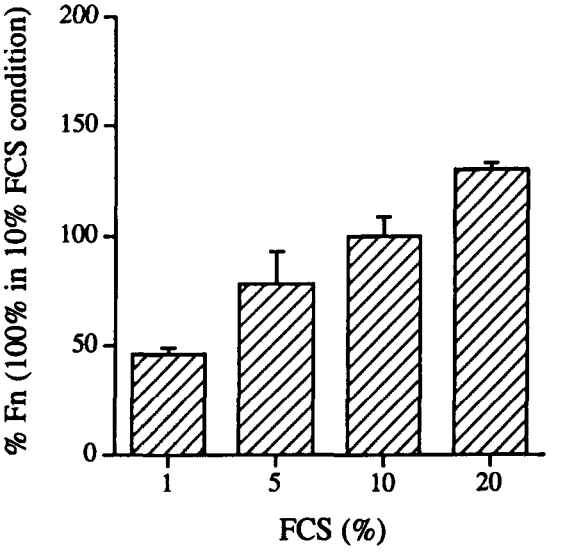

FIG. 8. Effect of serum concentration on fibronectin production by fibroblasts in 3-D culture. HFL-1 fibroblasts (1 × 10^5) were cultured in 0.5 mg/ml collagen gels in the presence of 1, 5, 10, and 20% of FCS. Fibronectin production was calculated by the difference between Day 1 and Day 2 cultures and expressed as ng fibronectin DNA in order to normalize to cell number as described above. *Vertical axis*: per cell fibronectin production expressed as a percentage of that of cells cultured in 10% fetal calf serum. *Horizontal axis*: serum concentration. The higher the concentration of FCS used, the more fibronectin was produced ($P < 0.01$, by ANOVA). Values are means of triplicate plates with *error bars* representing standard error.

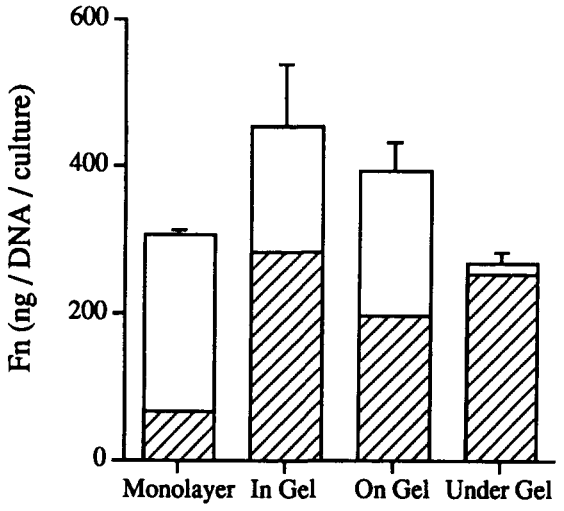

FIG. 9. Effect of cell position in culture on fibronectin production. Fibroblasts were cultured in monolayer culture or in three different relationships to the collagen gel: in, on, or under the gel (as described in "Materials and Methods"). Fibronectin production was calculated by the difference between Day 1 and Day 2 and expressed as ng fibronectin DNA in order to normalize to cell number as described above. Distribution of fibronectin between the culture media (*open bars*) and the cell layer and/or gel (*hatched bars*) depended on the culture condition. *Vertical axis*: fibronectin production normalized to DNA. *Horizontal axis*: culture conditions. Total fibronectin levels from the "under gel" was similar to those from "monolayer" culture. Total levels from the "on gel" was between those from the "in gel" and "monolayer" culture. Values are means of triplicate plates with *error bars* representing standard error.

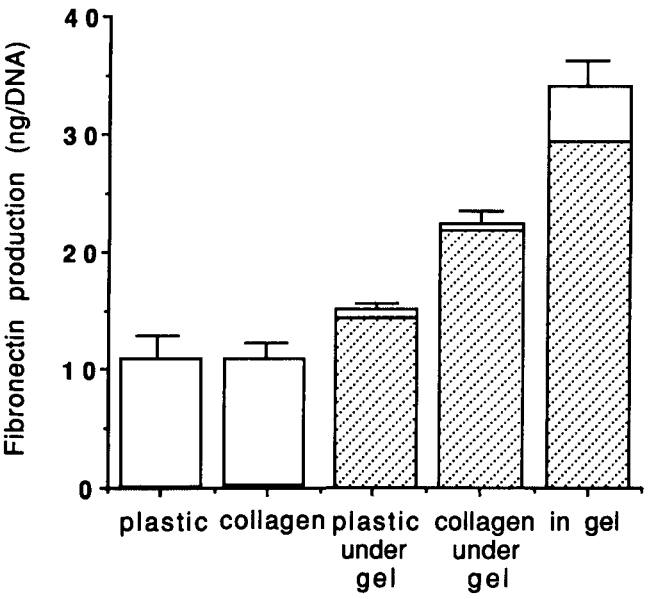

FIG. 10. Effect of plating fibroblasts on collagen-coated dishes on fibronectin production. Fibroblasts were plated on control dishes or on dishes which had been coated with a thin layer of type I collagen as described in "Materials and Methods." In some cases, the cells were then overlayered with a native type I collagen gel. For comparison purposes, cells were also cultured embedded in collagen gels as described in "Materials and Methods." Cells were then cultured and fibronectin production was quantified. *Vertical axis*: fibronectin production normalized to DNA; *horizontal axis*: culture conditions. *Open bars* indicate fibronectin in supernatant medium; *cross hatched bars* indicate fibronectin contained in the cell layer and gel. Culture of fibroblasts on collagen-coated dishes did not alter fibronectin production compared to culture on plastic under routine conditions. There was a slight increase in fibronectin production when cells were cultured on collagen under a collagen gel, but this was less than the fibronectin produced when cells were cultured embedded in the collagen gel.

In addition to increased fibronectin production, the distribution of the fibronectin produced in 3-D culture was different from that of monolayer culture. In monolayer culture, more than half of the fibronectin was released into the culture media. In 3-D culture, however, approximately two-thirds of the fibronectin stayed in the collagen gel. Direct measurements of the flux of fibronectin from a collagen gel into supernatant media were made. With gels made from 0.5 mg/ml collagen, the fibronectin equilibrated over about 3 d time. Equilibration was much slower with increasing collagen concentration, suggesting that the fibronectin was binding reversibly to the collagen in the gel and thus its diffusion was retarded. The retention of two-thirds of the newly synthesized fibronectin in the gel is consistent with such a process, although other mechanisms for retention are not excluded. The ability of the collagenous matrix to retain fibronectin could result, at least transiently, in locally high fibronectin concentrations. Such concentrations could, in turn, affect the biological properties of the matrix.

The experiments performed in the current report were performed in the presence of serum. Serum contains a large number of potential growth regulatory factors including some, such as fibronectin, which can interact with extracellular matrix. It is possible, therefore, that interaction of serum-derived factors with the collagen gel contributed to the stimulation of fibroblast fibronectin production observed in 3-D gel culture. Serum was also included in routine "dish" culture, but it is possible that the 3-D matrix altered the biologic effect of serum-derived mediators. Finally, the gels used in the current experiments were prepared from rat tail tendon collagen by a method that yields relatively pure type I collagen. *In vivo*, the 3-D matrix in which fibroblasts are embedded is much more complex. How a 3-D matrix containing other components would alter fibroblast behavior remains an interesting and important question.

Not only can the 3-D culture affect fibroblasts, but these cells can modify their surrounding matrix. Fibroblasts cultured in a 3-D gel are well known to cause retraction of the gel (7). This process of retraction is more marked when the collagen gel is detached from the supporting plastic dish and allowed to "float." Fibroblasts cultured in such "floating gels" are associated with a reduction in DNA (21,28), collagen, and total protein synthesis (17,22) which correlates with the degree of contraction (23,32). The current study was performed with gels which were attached to the supporting plastic dish in order to minimize retraction. Furthermore, the cultures were studied over a relatively brief time span. When the gels were intentionally detached and allowed to float and retract freely, fibronectin production was reduced. This suggests that the increased fibronectin production observed in the present study, in contrast to the reduced synthetic capacity observed in previous studies, was due to the culture conditions which reduced retraction. Consistent with this possibility, it has been reported that attached gel cultures have increased DNA synthesis compared to floating gel cultures (20), results we have confirmed. It remains an interesting possibility, therefore, that the mechanisms by which matrix alters fibroblast fibronectin production

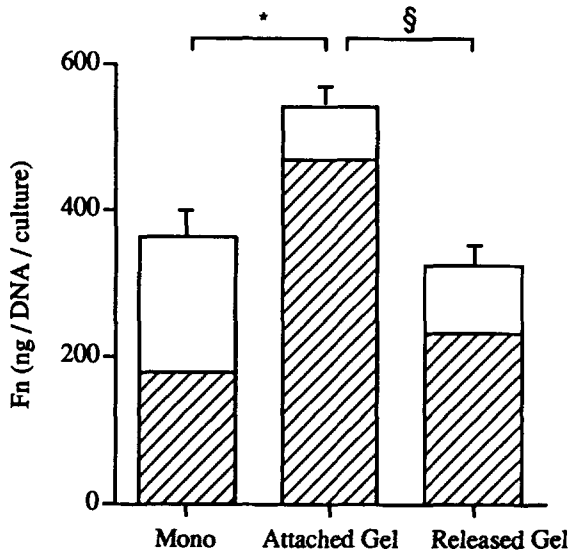

FIG. 11. Fibronectin production by fibroblasts in attached and released 3-D collagen gel culture. HFL-1 fibroblasts were cast into 0.5 mg/ml collagen gels in the presence of 10% FCS. One d later, "released gels" were released from the surface of the culture plates as described in "Materials and Methods." Fibronectin production was calculated by the difference between Day 1 and Day 2 cultures and expressed as ng fibronectin/mg DNA in order to normalize to cell number as described above. The results were compared with those in attached 3-D collagen gel cultures. *Open bars* represent the fibronectin released into the media, and *hatched bars* mean the fibronectin present in the collagen gel/cell layers. *Vertical axis*: fibronectin production normalized to cell number as DNA. *Horizontal axis*: culture condition. Fibronectin production by fibroblasts in the released gels was significantly lower than in the attached gels (*, $P < 0.05$, §, $P < 0.01$). Values are means of triplicate plates with *error bars* representing standard error.

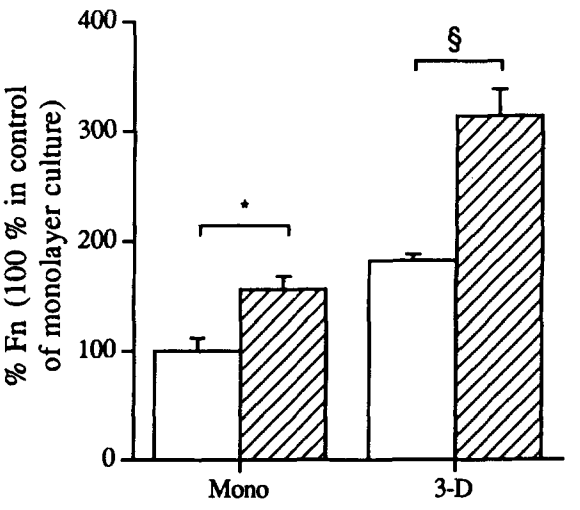

FIG. 12. Effect of TGF-β on fibronectin production in 3-D culture. Cells were plated in monolayer or 3-D cultures. After 1 d culture, cells were cultured with (*hatched bars*) or without (*open bars*) 100 p*M* of TGF-β for one more d in the presence of 10% FCS. Fibronectin production was calculated by the difference between Day 1 and Day 2 cultures and expressed as ng fibronectin DNA in order to normalize to cell number as described above. *Vertical axis*: fibronectin production normalized to DNA and expressed as a percentage of that produced by fibroblasts cultured in control conditions in monolayer culture. *Horizontal axis*: culture condition. *Crosshatched bars* = +TGF-β. The augmentation of fibronectin production by TGF-β in 3-D culture was similar to that in monolayer culture. In both conditions, TGF-β significantly increased fibronectin production of fibroblasts (*, $P < 0.05$, §, $P < 0.01$). Values are means of triplicate plates with *error bars* representing standard error.

involves both receptor binding and mechanical effects such as tensegrity (15,34).

Collagen retraction is thought to be a regular feature of scar remodeling (12). The present study demonstrates that fibroblasts can produce increased amounts of fibronectin in a collagen gel. This fibronectin may contribute to the subsequent fibroblast-mediated contraction of the collagen gel (3,11). Following contraction, protein synthesis by fibroblasts is reported to decrease (19). The ability of fibroblasts to modulate synthetic capacity may play an important role in the regulation of fibrotic and scarring processes.

The mechanisms by which 3-D collagen gel culture leads to increased fibronectin production are not established. Cells in 3-D culture are relatively well spaced in contrast to the close contacts observed in monolayer culture. While cell–cell contacts in 3-D culture cannot be excluded, cell density in the gel did not appear to affect fibronectin production. This contrasts with reports that fibronectin production in monolayer culture varies with cell density (24) and raises the possibility that cell–cell contacts might play some role in regulating fibronectin production. A number of alternative mechanisms could also play a role including the autocrine or paracrine production of soluble mediators.

There are several potential mechanisms by which cells could interact with the collagen matrix. Direct binding of fibroblasts to collagen, for example, contributes to fibroblast-mediated gel contraction (29). Alternatively, fibroblasts could interact with the collagen gel indirectly through fibronectin or other macromolecules (2,13). Finally, it is not possible, in the present study, to distinguish to what degree the effects are mediated by the collagen in the gel or by its 3-D nature independent of composition. The present study does not define the signal transduction pathway(s) which mediate matrix alterations of fibronectin production. Such pathways could include direct mechanical coupling of the extracellular matrix to the cellular cytoskeleton (15,34).

The observation that fibronectin production increases with increasing collagen concentration suggests that interaction of specific cell receptors with components of the extracellular matrix may play a role. The spatial architecture of the collagen, however, also seems to play a role because fibroblasts plated beneath a collagen gel behaved as if they were plated on plastic regardless of the collagen concentration in the gel. When cells were plated on top of a gel, results were intermediate, perhaps because a few of the cells migrated into the gel and were therefore in 3-D culture. Similarly, when cells were plated on a thin layer of type I collagen beneath the collagen gel, fibronectin production was also less than that in the collagen gel. Interestingly, with time, the cells progressively migrated into the collagen gel and the difference was lost. These results suggest that the 3-D architecture rather than the mechanism of cell adhesion was responsible for altered fibronectin production. It remains possible, however, that the fibroblasts interact differently with the air-dried, thin layer collagen or with collagen at the gel–liquid interface than they do with native fibers in a gel structure.

In the current series of experiments, there was no detectable difference in fibronectin mRNA levels between fibroblasts cultured on

plastic dish and in 3-D collagen gel by Northern blot analysis. Because the increase in total fibronectin measured by ELISA was about twofold in 3-D cultures, an effect mediated entirely by altered mRNA levels should have been detected. Although such a mechanism is not conclusively excluded, an effect at the level of translation seems more likely.

In summary, the present study demonstrates that fibroblasts in 3-D culture synthesize more fibronectin than those in monolayer culture. While the mechanism for this increase is not fully defined, increases in collagen concentration increases fibronectin production, and suspension of the fibroblasts in a 3-D matrix appears to be required. The fibronectin released from fibroblasts in 3-D culture diffuses slowly from the local environment. These interactions between fibroblasts, their secreted products, and the surrounding matrix could play important roles in tissue remodeling in development of scar formation and in fibrosis.

ACKNOWLEDGMENTS

The authors would like to acknowledge with gratitude the technical support of Mary G. Illig, Peggy Heires, and Art Heires, and the secretarial support of Lillian Richards. We also thank Dr. Yoko Adachi for her helpful suggestions regarding this manuscript.

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