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Modulation of Collagen and Fibronectin Synthesis in Fibroblasts by Normal and Malignant Cells

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Abstract The influence of various normal and malignant human cells on the level of collagen synthesis by human fibroblasts was tested in coculture. As revealed by immunoperoxidase staining, in cocultures with breast adenocarcinoma cells (MCF7, SA52, T47D) fibroblasts synthesized collagen while tumor cells did not. Fibroblasts displayed increased collagen production without change in the overall protein synthesis. Several other types of cells derived from normal human tissues (keratinocytes, normal mammary cells) or from fibrosarcoma, melanoma, cervical carcinoma, choriocarcinoma, or other breast adenocarcinoma (SW613, MDA, BT20) did not affect collagen synthesis of fibroblasts. Although to a lesser extent, this stimulating effect was reproduced by using the conditioned medium (CM) of the active cells but not with CM of the other cell types. A slight stimulation was also obtained when tumoral MCF7 cells and fibroblasts shared the same medium but were physically separated, suggesting that close contact was required for optimal stimulation of collagen synthesis. The collagen synthesis stimulating activity was not related to a modification of fibroblast proliferation rate. The production of collagen types I, III, and VI and fibronectin were increased in cocultures of fibroblasts with MCF7 cells. The increased synthesis of collagen types I and III and fibronectin was paralleled by similar changes in the steady-state level of their mRNAs. On the contrary, the increased production of collagen type VI appeared regulated at a post-transcriptional level.

Key words: tumor cells, cell-cell interaction, desmoplasia, extracellular matrix, stroma reaction

Several human tumors (melanoma and breast, prostate, pancreatic and gastric colon carcinoma) are often associated with a fibrotic reaction or desmoplasia. Accumulation of connective tissue around the growing tumor mainly consists of various types of interstitial collagen, fibronectin, elastin [1–5] and proteoglycans [6–9]. This modification of the extracellular matrix in contact with invasive carcinoma can take place by distinct mechanisms. The first might be the consequence of the degradation and/or synthesis of matrix components by invading tumor cells. The second might result from a modulation of the host fibroblast's phenotype by the presence of tumor cells stimulating either proliferation [4,10] or synthesis of matrix components. Several growth factors are known to modulate the biosynthetic activity of mesenchymal cells as TGFα, TGFβ, PDGF, FGF, and EGF [11–15]. Tumor cells have been shown to secrete a variety of these cytokines [16] and other soluble factors able to specifically increase glycosaminoglycans [7–9] or collagen synthesis by fibroblasts [17]. Their activity on the stromal cells might operate at various steps including the transcriptional control, the stability of the mRNA, the rate of the mRNA translation, or a modulation of the intra- or extracellular degradation of structural components.

In this report, we present evidence that several, though not all, human mammary adenocarcinoma cells stimulate collagen and fibronectin synthesis by normal human skin fibroblasts in coculture. This activity is mediated by a soluble factor released by the cells in the medium and is not related to a modification of the proliferation rate of fibroblasts. It operates at least in part by modulating the steady-state level of specific mRNAs.

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MATERIAL AND METHODS

Cell Culture

Human mammary tumor cell lines MDA-MB 231 [18], SA52 [19], and SW613 [20] were provided by M.F. Poupon (Centre de Recherche sur le Cancer, Villejuif, Paris, France); MCF7 [21] was provided by G. Leclercq (Institut Bordet, Bruxelles); BT20 and T47D [22] were supplied by R. Gol (University of Liège, Belgium). The A431 cervical carcinoma cells, B16 murine melanoma cells, and human choriocarcinoma Bewo cells lines were obtained from the American Type Culture Collection (Rockville, MD, USA). MCF7 cells stably transfected with the transforming DNA from Harvey murine sarcoma virus and selectable marker pSV2 gpt (MCF7 ras) [23] were a kind gift of M. Lippman (Bethesda, MD). A line transfected with pSV2 gpt was used as control (MCF7 gpt). Human fibrosarcoma HT1080 cells [24] were supplied by M. Aumailley (Max Planck Institut für Biochemie, Munich, Germany).

Normal fibroblasts were obtained by outgrowth from human skin explants as previously described [25] and used between passages 4 and 14. Keratinocytes were collected from human foreskin and cultured in 75% DMEM (Gibco), 25% HAM F12 supplemented with 10% decomplemented FCS, hydrocortisone (0.4 μg/ml), cholera toxin (10^{-10} M), insulin (5 μg/ml), adenine (20 μg/ml), transferrin (5 ng/ml), triiodothyronine (1.5×10^{-5} ng/ml), and epidermal growth factor (EGF, 5 ng/ml) on mitomycin treated Balb 3T3 as described by Green et al. [26].

Normal epithelial mammary cells (NMC) were obtained from reduction mammoplasty and processed immediately after surgery as previously described [27,28]. Primary cultures were performed in a low Ca^{++} concentration medium (adjusted to 0.06 mM Ca^{2+} with $CaCl_2$)—that is, Joklik-modified Eagle's medium supplemented with 10% fetal calf serum (FCS) lowered in Ca^{++} by Chelex treatment, glutamine (2 mM), nonessential amino acids, insulin (10 μg/ml), cholera toxin (100 ng/ml), cortisol (0.5 μM), EGF (20 ng/ml), and fungizone (5 μg/ml). Culture medium was changed every three days and the primary cells used in the experiment were collected by trypsin treatment of a 15-day-old culture.

Human fibroblasts, B16 cells, MCF7 cells, SA52 cells, SW613 cells, BT20 cells, and MDA-MB 231 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% FCS, penicillin-streptomycin (100 U/ml), and ascorbic acid (50 mg/ml). Bewo cells were cultured in HAM F12 (Gibco) supplemented with 15% FCS. The T47D cells were maintained in RPMI 1640 (Gibco) containing 10% FCS.

Hormone Treatment

Estradiol (10 nM) (E2) (Merck, Germany) was solubilized in ethanol and added to the culture medium at a maximum final concentration of ethanol of 0.1%. Medium was changed every two days. Tumoral cells (MCF7 cells or MCF7 gpt cells) were pretreated with estradiol or ethanol alone for one week before use in monoculture or coculture with fibroblasts.

Preparation of Conditioned Medium

Cells were cultured with 10 ml of their respective medium in the absence or presence of serum. After 2 days, the conditioned medium was harvested, centrifuged to remove cell debris, and stored at 4°C.

Quantification of Collagens and Fibronectin

Collagen types I and III were purified as previously described [29] and collagen type VI was extracted from human placenta by pepsin digestion and purified according to Odermatt et al. [30]. The purity of these preparations was tested by SDS-PAGE [31], Western blot [32], and ELISA [33].

Antiserums directed against types I, III, and VI collagen and fibronectin were raised in rabbit and guinea pigs. The specificity of the antibodies was tested by Western blot [32], ELISA [33], and radioimmunoblotting assay [34].

The level of synthesis of various types of collagen and fibronectin was measured by a radioimmunoblotting assay using the method of Hatamoshi et al. [34]. The cell layer was rinsed with phosphate buffered saline (PBS) and scraped in 6 M guanidinium hydrochloride buffered with 50 mM Tris, 0.15 M NaCl at pH 7.4 (Tris Buffered Saline, TBS). The collagen secreted in the medium was collected by centrifugation of a 40% ammonium sulfate precipitation and the precipitate solubilized in 6 M guanidinium hydrochloride buffered as above. Serial dilutions of these samples were dot blotted on nitrocellulose filters. Serial dilutions of purified collagens in

the same solution (from 0.1 to 20 μg) were blotted on the same filter and used as standards. After washing with TBS, filters were saturated with 2.5% BSA in TBS overnight at 4°C. Incubation with specific antibodies was performed for 2 hours at room temperature in TBS-BSA. The filters were washed 2 times and incubated with ^{125}I-protein A (5 μCi/20 ml) for 2 hours. After extensive washings and drying, the filters were exposed to Royal-S-O-Mat Kodak film. The signal intensities were recorded by scanning LASER densitometry (Ultroscan XL, LKB).

Immunohistochemical Procedure

The endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol and non-specific staining was prevented by use of normal swine serum. The first rabbit antibody to type I collagen or fibronectin was added at a dilution of 1:40 and incubated for 1 hour at 37°C. After washing in TBS, cells were incubated for 30 minutes with swine anti-rabbit immunoglobulin (Prosan, Ghent, Belgium) diluted 1:40 in the same buffer containing 1% BSA. After washings, cells were incubated for 30 minutes with the rabbit peroxidase-antiperoxidase complex (Prosan). The antigens were visualized using 3,3'-diaminobenzidine tetrahydrochloride (DAB) for 10 minutes. Hematoxylin was used as counterstain.

Biosynthetic Studies

In a typical experiment, 10^5 fibroblasts and/or tumor cells were plated on plastic in medium supplemented with 10% dialysed and decomplemented FCS. After 1 day of culture, the medium was replaced by freshly prepared medium containing ^{3}H-proline (10 μCi/ml) and ascorbic acid (50 μg/ml) and metabolic labeling performed for 24 hours. Coculture of fibroblasts and primary NMC was performed either in Joklik medium as above or in DMEM, in absence of cholera toxin. Similar results were obtained in both mediums. In some experiments, 3 ml of medium conditioned by tumor cells were added to 2 ml of fresh medium at the beginning of the metabolic labeling of fibroblast cultures.

The overall protein synthesis and collagen synthesis was determined by measuring peptide bound ^{3}H-proline and ^{3}H-hydroxyproline by the method of Juva and Prockop [35]. Collagen synthesis was also determined by measuring the radioactivity incorporated into collagenase-sensitive proteins [36] using highly purified bacterial collagenase (form III, Advance Biofactures, NY, USA).

Analysis of labeled collagen polypeptides was performed after precipitation with 40% ammonium sulfate [37]. Samples containing 5×10^4 cpm were electrophoresed in 6.25% polyacrylamide slab gels [31] under non-reducing and reducing conditions. Fluorography was carried out according to Bonner and Laskey [38] using S-O-Mat Kodak X-ray films.

Cell Number and Rate of Multiplication

Proliferation of epithelial cells and fibroblasts was measured in monoculture and in coculture at a cellular ratio of 1/1. In some assays, cells were treated for 2 hours with mitomycin (10 μg/ml) and washed before plating the other cell type. Cells were labeled for 6 hours with 7 μCi/ml of ^{3}H-thymidine (methyl ^{3}H-thymidine; 6.7 μCi/mmol; NEN). The radioactive material precipitated by addition of trichloracetic acid (TCA) (30%) was collected on Whatman GFA filters and measured by liquid scintillation spectrometry in an LKB 1217 Rack Beta. The DNA content of sonicated cells suspension was determined by fluorimetry using the bis-benzimidazol H 33258 reagent (HOECHST, S.A., Brussels, Belgium) [39].

Measure of the Steady-State Level of Specific mRNA

The cDNA probes specific for alpha 1(I) (Hf-677) [40] and alpha 2(I) (Hf-32) [41] collagen chains were kindly provided by Dr. F. Ramirez (New York, USA) and for alpha 1(III) (pIII-33) [42] by Dr. R. Crystal (Bethesda, MD, USA). The alpha 1(VI), alpha 2(VI), and alpha 3(VI) [43] cDNA probes were a kind gift of Dr. R. Timpl (Munich, Germany). The cDNA probe specific for fibronectin [44] was a gift of Dr. M. Sobel (Bethesda, MD, USA).

Labeling of the probes with ^{32}P. dCTP (3,000 Ci/mmol, Amersham) was performed using a random priming DNA labeling kit (Boehringer) as described by the manufacturer. Probes were separated from unincorporated radionucleotides by filtration through a column of Sephadex G50.

The cell layer was washed with PBS and treated with a lytic solution (RNA Zol) (Cinna/Biotecx, Texas, USA). Total RNA was isolated as described by Chomczynski and Sacchi [45].

For Northern analysis, heat denatured RNA in 50% formaldehyde was electrophoresed in 1% agarose and capillary blotted into nylon mem-

branes (Hybond N, Amersham) as described by Fourney et al. [46].

For dot blot analysis, total RNA was heat denaturated in a solution of 13% formaldehyde and 10 × standard saline citrate (SSC), serially diluted and slot blotted on nylon membrane using a Schleicher and Schull manifold. Prehybridization and hybridization with specific cDNAs were performed as described by Maniatis et al. [47]. The nylon membranes were washed twice at room temperature in 2 × SSC, 0.1% SDS and 6 times at 65°C with progressively decreasing concentrations of SSC, 0.1% SDS (2 × SSC to 0.3 × SSC). The membranes were exposed to Kodak S-O-Mat films. The intensity of the signals was quantified by LASER scanning densitometry (Ultroscan XL, LKB). For each experiment, the amount of total slotted RNA was controlled by hybridization at 60°C, with a 28 S rRNA specific oligonucleotide probe as described by the manufacturer (Clontech Laboratories, USA).

RESULTS

Effect of Various Epithelial Cells on Collagen Synthesis by Fibroblasts in Coculture

Collagen type I was localized by immunoperoxidase technique in coculture of fibroblasts and MCF7 cells. Fibroblasts synthesized collagen while tumoral MCF7 cells did not (Fig. 1). Quantification of collagen synthesis was performed by radiolabeling with ^{3}H-proline and measurement of ^{3}H-hydroxyproline. In monoculture, normal mammary cells (NMC) and breast adenocarcinoma MCF7 cells synthesized minimal amounts of collagen while in fibroblast cultures, collagen accounted for 16–20% of the proteins secreted in the medium and for 1% of the proteins in the cell layer. In coculture of fibroblasts and NMC at a cell ratio of 1 to 1, total protein and collagen synthesis were the sum of the individual production observed in monocultures (Table I). On the opposite, at an identical cellular ratio of fibroblasts to tumoral MCF7 cells, collagen synthesis was significantly higher in both the cell layer and in the medium while total protein synthesis was not affected (Table I). When a constant number of fibroblasts was incubated with an increasing number of MCF7 cells, the enhancement of the collagen production was found to be related to the number of MCF7 cells (Fig. 2).

The capacity of various normal and tumoral cells to stimulate fibroblast collagen synthesis was investigated in coculture. In monoculture, except fibroblasts, none of these cells synthesized collagen (Table II). In coculture, five human breast adenocarcinoma cell lines (SA52,

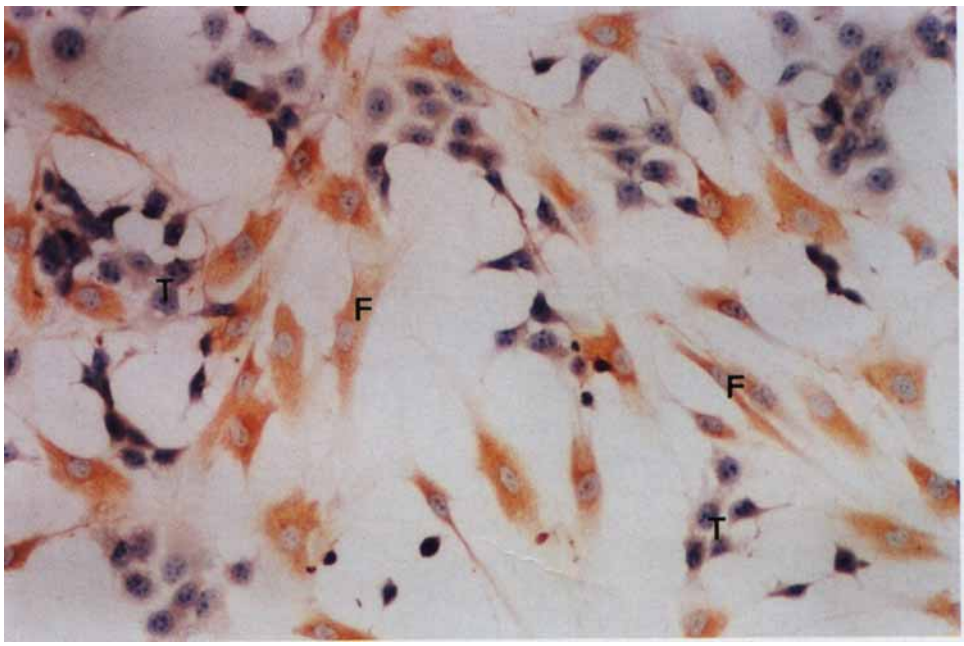

Fig. 1. Type I collagen immunostaining of tumoral MCF7 cells (T) cocultured with fibroblasts (F). Fibroblasts synthesizing collagen were stained while tumoral cells were not.

TABLE I: Protein and Collagen Synthesis in Mono- and Coculture of Fibroblasts (F) and Normal (NMC) or Tumoral (MCF7) Mammary Cells*

	Protein synthesis (cpm × 10^3/dish)			Collagen synthesis (cpm × 10^3/dish)		
	Cell layer	Medium	Total	Cell layer	Medium	Total
F	68	142	210	10.7	50.0	60.9
NMC	49.5	436	486	0.8	2.5	3.0
F + NMC	102	620	722	9.5	44.0	54.0
F	72	120	183	10.5	58.6	69.0
MCF7	31.5	661.5	693	0.9	3.4	4.0
F + MCF7	90	800	900	26.0	143.0	169.0

*Fibroblasts, NMC, and MCF7 were cultured alone and cocultured for 48 hours at an initial cell ratio of 1:1 as described in Materials and Methods. Collagen synthesis was determined by measuring non-dialyzable ^{3}H-hydroxyproline and total protein synthesis by measuring total nondialyzable radioactivity. The results are calculated for an initial seeding of 3.10^5 cells in monoculture and 3.10^5 for each cell type in the cocultures. Total collagen production (i.e., cell layer associated collagen and collagen released in the medium) was stimulated in coculture with tumoral cells by a factor of about 2.5 but not in coculture with normal NMC cells. Coculture had no effect on total protein synthesis.

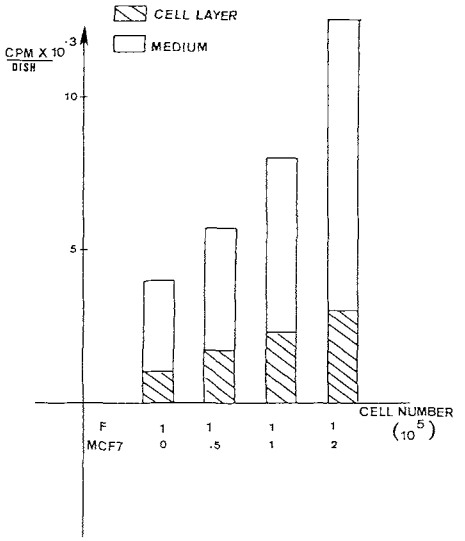

Fig. 2. A constant number of human skin fibroblasts (F = 10^5 cells) was cocultured on plastic with increasing number of MCF7 cells (0 to 2 × 10^5 cells). Collagen synthesis was determined in the medium and in the cell layer by measuring non-dialyzable ^{3}H-hydroxyproline by the technique of Juva and Prockop [35]. The stimulation of collagen production was related to the ratio of MCF7 cells to fibroblasts and occurred both in the cell layer and the medium.

T47D, MCF7, MCF7 Ras, and MCF7 gpt) stimulated the production of collagen by fibroblasts (Table II). None of these malignant cells influenced the distribution of newly synthesized collagen between the medium and the cell layer (data not shown). Other breast carcinoma cell lines (BT20, MDA, SW613), human choriocarcinoma cells (Bewo), melanoma B16 cells, cervical carcinoma cells (A431), fibrosarcoma cells (HT1080), or normal keratinocytes did not influence collagen synthesis by fibroblasts. The extent of collagen stimulation was similar in cocultures of fibroblasts with untreated or estradiol-treated MCF7 cells, control MCF7 gpt or MCF7 ras cells (Table II). The ability of MCF7 cells to respond to estrogen was verified by performing an estradiol-dependent proliferation test by ^{3}H-thymidine incorporation (data not shown). As the net increase in collagen production could depend on a stimulation of fibroblast proliferation, cells cultured separately or cocultured were labeled with ^{3}H-thymidine for 6 hours. The incorporation of ^{3}H-thymidine in coculture (18,500 cpm ± 4,000/culture) did not significantly differ from the sum of the radioactivity incorporated in monoculture of fibroblasts (400 cpm ± 90) and MCF7 cells (13,000 cpm ± 2,500). Furthermore, the thymidine incorporation and the number of fibroblasts determined by DNA content were unchanged when mitomycin-treated MCF7 cells were added to fibroblasts (Fig. 3a,b). The stimulation of collagen production by MCF7 cells persisted when mitomycin-treated fibroblasts and mitomycin-treated or untreated MCF7 cells were cocultured (Fig. 4).

Characterization and Quantification of Newly Synthesized Collagens

The labeled collagen polypeptides recovered from culture medium or cell layer were characterized by SDS-polyacrylamide gel electrophoresis under reducing and non-reducing conditions. Similar electrophoretic patterns were observed

TABLE II. Collagen Synthesis in Monoculture or Coculture of Various Normal and Tumor Cells With Fibroblasts*

Cell types	Collagen synthesis (103 cpm/dish) Mono-culture	Coculture with fibroblasts	Collagen stimulating capacity
Normal cells			
fibroblasts	2.01 ± .15	–	–
NMC	.05 ± .02	1.7 ± .15	–
keratinocytes	.1 ± .03	1.5 ± .2	–
Tumoral cells			
Fibrosarcoma			
HT1080 cells	.1 ± .03	2.05 ± .08	–
melanoma			
B16	.05 ± .02	2.2 ± .12	–
choriocarcinoma			
Bewo	.1 ± .03	2.1 ± .3	–
cervical carcinoma			
A431	.07 ± .25	2.5 ± .2	–
Breast adenocarcinoma			
BT20	.2 ± .08	1.95 ± .35	–
MDA	.13 ± .08	1.9 ± .5	–
SW613	.04 ± .02	2.1 ± .3	–
SA52	.08 ± .03	3.9 ± .15	+
T47D	.04 ± .02	4.1 ± .3	+
MCF7	.05 ± .01	4.2 ± .2	+
MCF7 + E2	.04 ± .01	4.5 ± .1	+
MCF7 gpt	.06 ± .01	4.3 ± .2	+
MCF7 gpt + E2	.04 ± .01	4.5 ± .1	+
MCF7 ras	.05 ± .05	4.0 ± .25	+

*Fibroblasts (10^5 cells) and normal or tumoral cells were plated on plastic either in monoculture or in coculture at a cell ratio of 1:1. Collagen synthesis was determined in the cell layer as described in Material and Methods. In monoculture, only normal fibroblasts synthesized collagen. Collagen production by the same number of fibroblasts was increased by coculture with several breast adenocarcinoma cells (+) but remained unchanged with several other cell types tested (–). The values represent the average of 3 experiments and their standard deviation.

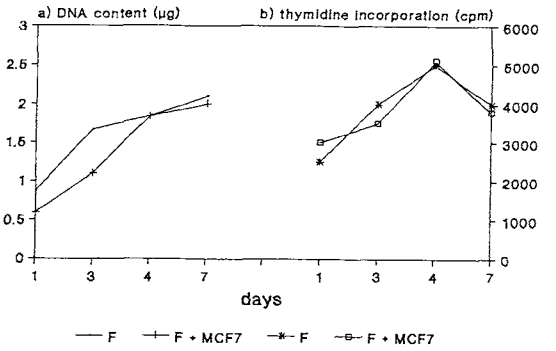

Fig. 3. Effect of coculture of fibroblasts and mitomycin-treated MCF7 cells on fibroblasts proliferation. Fibroblasts were either cultured alone or cocultured with MCF7 cells treated with mitomycin as described in Material and Methods. Proliferation was estimated by measuring DNA content (a) and ^{3}H-thymidine incorporation (b) as a function of time (days) in culture. The DNA content and ^{3}H-thymidine incorporation in the fibroblasts was calculated by subtracting from the total values the amount of DNA and ^{3}H-thymidine incorporation associated with mitomycin-treated MCF7 cells and maintained for the same period of time in monoculture.

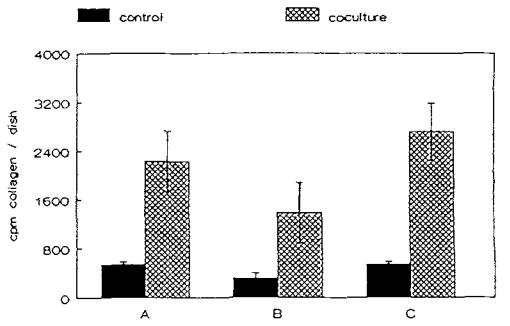

Fig. 4. Collagen synthesis determined in the cell layer of cocultures of (A) mitomycin-treated fibroblasts with untreated MCF7 cells; (B) untreated fibroblasts with mitomycin-treated MCF7 cells; (C) mitomycin-treated fibroblasts and mitomycin-treated MCF7 cells. Monocultures of mitomycin-treated (a,c) or untreated (b) fibroblasts were used as control.

in monoculture of fibroblasts and in coculture with MCF7 cells (Fig. 5). These polypeptides displayed the same electrophoretic mobility as those of authentic chains and were sensitive to bacterial collagenase. After a 24-hour labeling period, procollagen and partially processed p-C and p-N collagen were the predominant components. After pepsin digestion, the precursor forms were converted into polypeptides chains that co-migrated with the alpha 1 and alpha 2 chains of type I collagen, with the alpha 1(III) chains and with the alpha 1 and alpha 2 type (VI) collagen chains.

The various types of collagen and fibronectin in the medium and in the cell layer of fibroblasts and MCF7 cells cultured alone or cocultured were further quantified using radioimmunoblotting procedures. Cell layer extracts from fibroblasts and MCF7 cells cultured separately for the same period of time and then mixed before performing the measurements were used as control. The lack of collagen synthesis by MCF7 cells was again demonstrated by these immunological techniques. In the cell layer of cocultures, the amount of collagen types I and VI and fi-

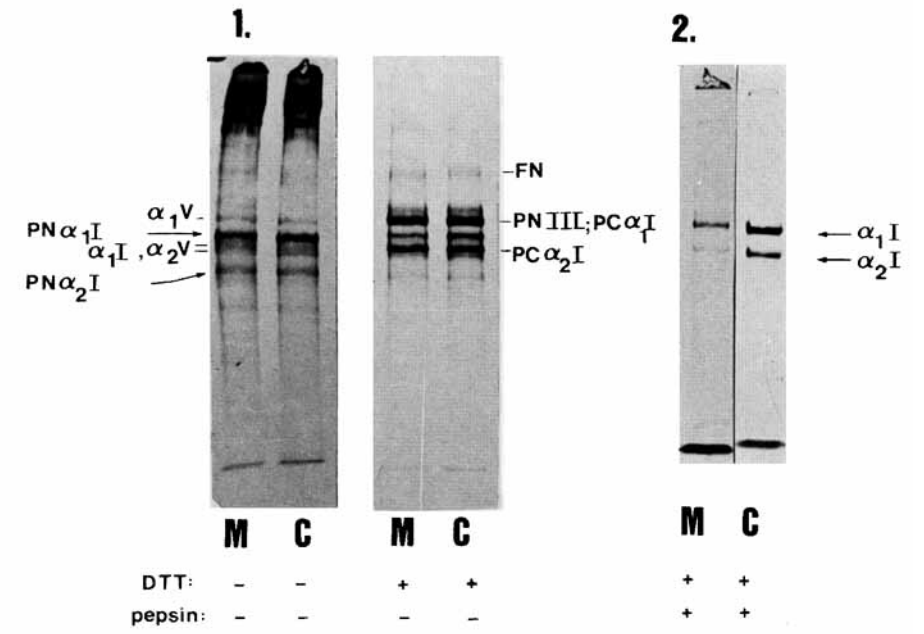

Fig. 5. Fluorography of ^{3}H-proline labeled proteins collected by a 40% ammonium sulfate precipitation from culture medium (1) or cell extracts digested with pepsin (2) of fibroblasts grown in monoculture (M) or in coculture (C). The electrophoresis was performed under non-reducing (−) and reducing (+) conditions. DTT, dithiothreitol.

bronectin approximately doubled as compared to monoculture of fibroblasts or to fibroblasts and MCF7 cells cultured separately and then mixed during cell layer extraction (Fig. 6, Table III). Collagen type III production was stimulated to a lesser extent (about 1.3 times). A similar increase of collagen secreted in the medium was observed (data not shown).

Measurement of Specific mRNA Levels

The steady-state level of mRNAs coding for type I, III, and VI collagens and fibronectin was analyzed by Northern blot and slot blot hybridization with specific cDNA probes. None of the cDNA probes used in Northern blot analysis reacted with MCF7 cells' mRNA. The RNA extracted from MCF7 cells did not modify the migration of the specific mRNAs extracted from fibroblasts or cocultures (Fig. 7a). Densitometric analysis of the slot blot autoradiograms (Fig. 7b) indicated a twofold increase in the steady-state level of alpha 1 type I, alpha 2 type I, and alpha 1 type III collagens and fibronectin mRNA in coculture of fibroblasts and MCF7 cells as compared to fibroblasts and tumoral cells cultured separately and mixed before RNA extraction (Table III). Separately cultured fibroblasts and fibroblasts in coculture exhibited similar levels of mRNA coding for type VI collagen alpha 1, alpha 2, and alpha 3. The increase of type I and type III collagens and fibronectin synthesis observed in coculture was then paralleled by an increase in mRNA levels while collagen type VI synthesis was not regulated at a pre-translational level.

Effect of Conditioned Medium of Tumoral Cells on Collagen Synthesis by Fibroblasts

In order to determine whether the stimulatory effect could be mediated by factors released by tumor cells, conditioned medium (CM) of MCF7 cells was tested on fibroblasts. Under these conditions, collagen synthesis was stimulated by a factor of 1.5 to 2.5. A similar increase was observed with CM of the other cell lines able to stimulate collagen synthesis by fibroblasts in coculture (MCF7 gpt, MCF7 ras, T47D, SA52) but not with the CM of the inactive cells. When medium was conditioned by increasing the number of MCF7 cells, collagen stimulation increased gradually as a function of the density of MCF7 cells up to a maximum value (Fig. 8). This maximal stimulation was reached at low MCF7 cell density (3×10^5 cells/dish). The release of the diffusible factor(s) by MCF7 cells was not influenced by the concentration of serum in the medium used for the preparation of CM (data not shown). The extent of collagen stimulation

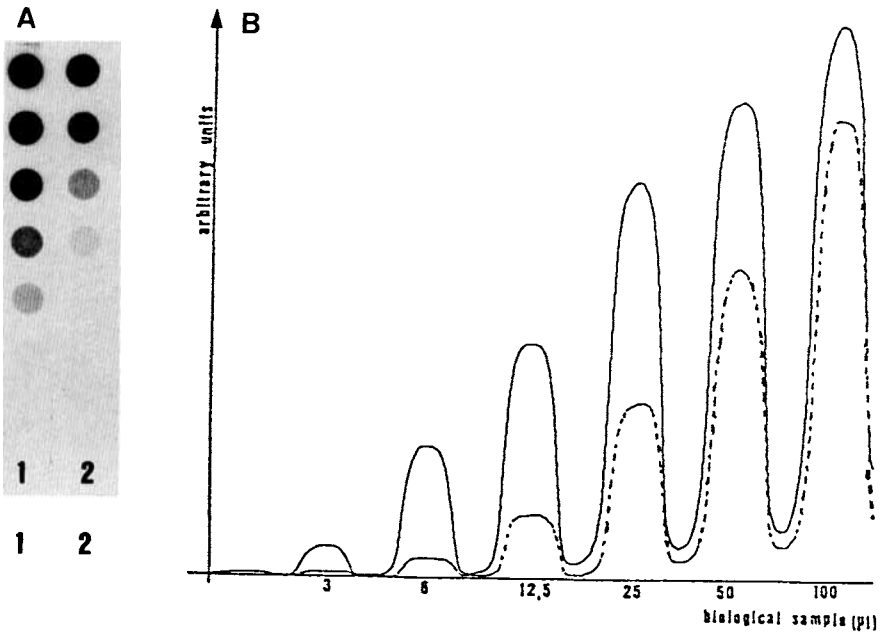

Fig. 6. Examples of immunological dot binding assay used for the determination of collagen type VI. **A:** Serial dilutions of cell extracts obtained from cocultures (1) or monocultures of fibroblasts and MCF7 cells pooled before extraction (2) were dot blotted and incubated with specific antiserum and ^{125}I-protein A as described in Material and Methods. **B:** Autoradiograms of protein dot blots were analysed by LASER scanning densitometry. The area under the peaks was quantified and found linearly related to the amount of cell extracts (µl). Continuous line, cocultures; dotted line, separate cultures of MCF7 cells and fibroblasts pooled before extraction.

was generally lower in the presence of CM than in coculture (1.5- to 2.5- vs. 2- to 5-fold). Similarly, when fibroblasts and MCF7 cells were cultured on distinct coverslips sharing the same medium,the stimulation of collagen production was lower (about 1.5-fold) than when the cells were cocultured on the same coverslip. This suggests that cell proximity between tumor cells and fibroblasts would be required for an optimal effect on collagen synthesis.

TABLE III. Variations (in %) Induced by Coculture as Compared to Monoculture of Fibroblasts in the Amount of Various Types of Collagen and Fibronectin and in the Steady-State Level of the mRNAs of These Proteins*

	Proteins	Probes	mRNA
Collagen types			
Type I	195 ± 35	alpha 1 (I)	200 ± 50
		alpha 2 (I)	202 ± 21
Type III	130 ± 7	alpha 1 (III)	214 ± 17
Type VI	260 ± 50	alpha 1 (VI)	91 ± 20
		alpha 2 (VI)	80 ± 20
		alpha 3 (VI)	85 ± 20
Fibronectin	200 ± 20		250 ± 20

*The amount of collagen and fibronectin in the cell layer was measured by radioimmunoblotting and the estimation of the steady-state level of specific mRNAs as described in Material and Methods. The results are expressed in percentage of the values recorded in the cocultures compared to the monocultures of MCF7 cells and fibroblasts pooled just before processing and taken as 100%. The values are the average of 3 separate determinations and the standard deviation.

DISCUSSION

Several human cancers are associated with an accumulation of a newly formed connective tissue known as the desmoplastic reaction. It is most frequently observed in breast tumors. Limited effort has been directed to elucidate the cellular and molecular mechanisms. It has yet to be established whether the accumulating matrix components are produced by host mesenchymal cells or by the tumor cells or if a series of complex interactions between these types of cells can explain this reaction. By electron microscopy, types I and III collagen have been shown to be present in the rough endoplasmic reticulum of fibroblasts and myofibroblasts of the fibrous stroma of gastric carcinoma [48]. In vitro studies have demonstrated that collagen synthesis is increased when fibroblasts are grown on a preformed breast tumor matrix [1] while the breast tumor cell-conditioned medium failed

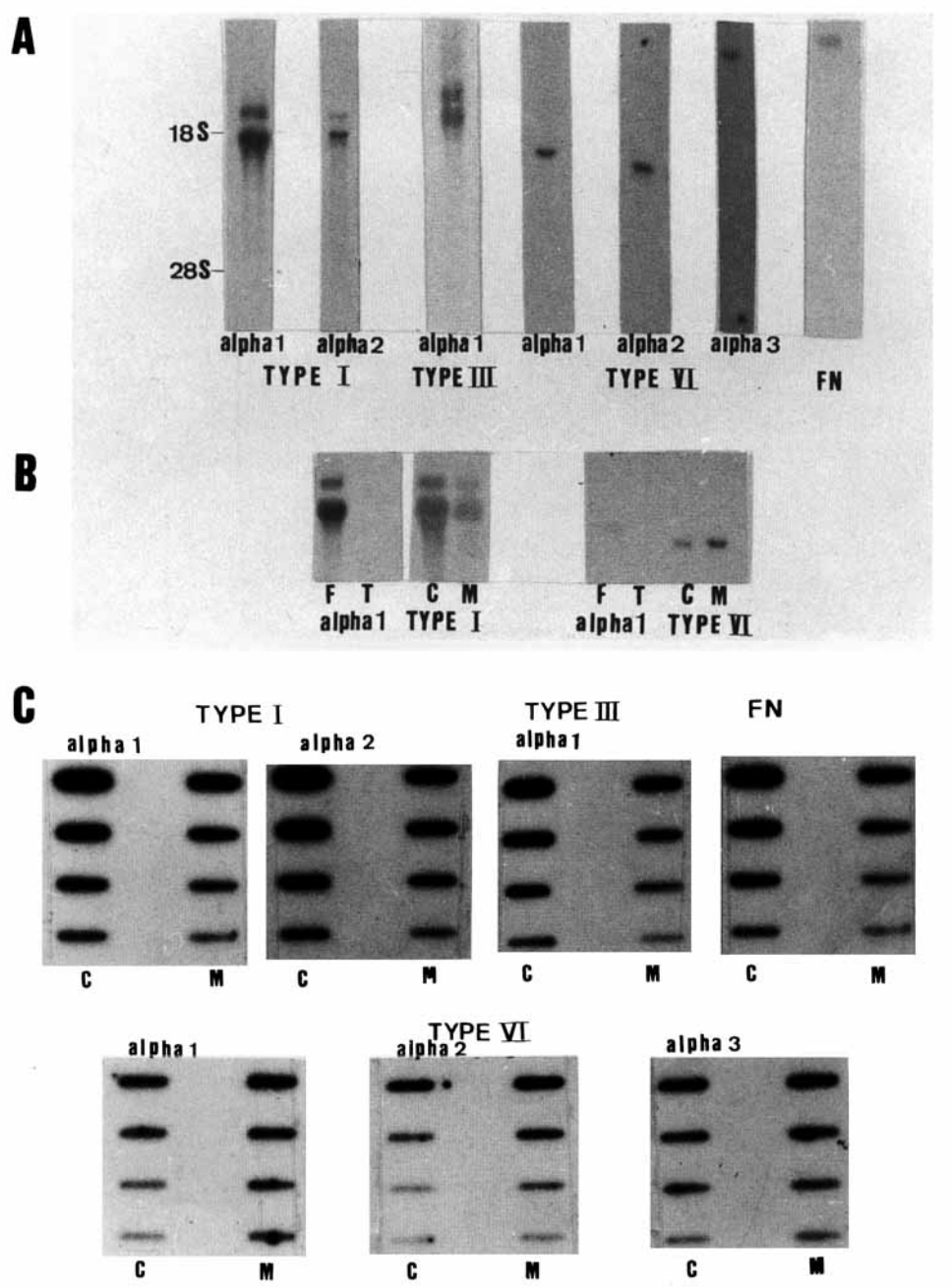

Fig. 7. RNA was extracted from monocultures of MCF7 (T), monocultures of fibroblasts (F), cocultures of fibroblasts and MCF7 (C), and monocultures of the two types of cells mixed before extraction (M). **A:** Northern blot analysis of RNA hybridized with alpha 1 type I, alpha 2 type I, alpha 1 type III, alpha 1 type VI, alpha 2 type VI, alpha 3 type VI, and fibronectin (FN) cDNA probes revealed the presence of transcripts showing the characteristic and specific hybridization pattern as previously described [40–44]. **B:** The tumoral MCF7 cells (T) did not express mRNA coding for these proteins as illustrated for alpha 1 type I and alpha 1 type VI chains. The mRNA level coding for alpha 1 type I collagen chain was increased in cocultures (C) as compared to the control (M). On the contrary, the mRNA level of alpha 1 type VI collagen chain was slightly decreased in cocultures. **C:** Quantification was performed on serial dilutions of total RNA (from 1 to 0.125 μg) slot blotted and hybridized with each indicated probes as described in Material and Methods.

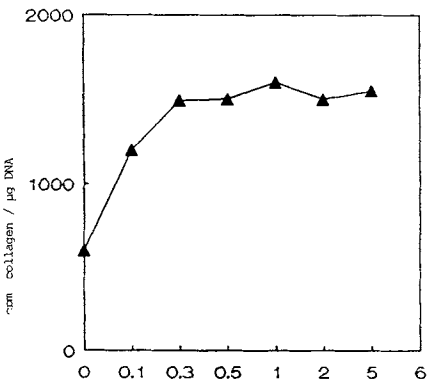

Fig. 8. Effect of conditioned medium of MCF7 cells on collagen synthesis by fibroblasts (10^5). The medium was conditioned by increasing the number of MCF7 cells. Collagen synthesis was measured by radiolabeling as described in Material and Methods. The stimulation of collagen synthesis is related to the density of the MCF7 with a maximal effect reached at 3×10^5 cells/dish.

to display the same effect. These studies suggest that, in vivo, collagen of the desmoplastic matrix surrounding carcinoma is produced by connective tissue cells. The present study demonstrates that in cocultures, human fibroblasts are induced by human breast carcinoma cells (MCF7) to produce increased amounts of collagen and fibronectin. This effect is related to the transformed phenotype of the cells, since normal mammary epithelial cells, as well as normal keratinocytes, showed no such capacity. In a series of tumor cells, only some strains of breast adenocarcinoma (MCF7, MCF7 ras, MCF7 gpt, T47D, SA52) displayed the stimulating property. Tumor cells derived from fibrosarcoma, melanoma, choriocarcinoma, and cervical carcinoma were inactive. It is interesting to note that the desmoplastic reaction in human breast cancer is mostly observed in infiltrating ductal and lobular carcinoma [1,2]. Several cell functions (proliferation, biosynthetic activity, etc.) of breast adenocarcinoma cells are under hormonal control that can be bypassed by v-Ha-ras transfection. However, no correlation was observed between estradiol responsiveness and the collagen stimulative capacity. Estradiol-independent (MCF7 ras) as well as estradiol-dependent (MCF7 gpt, T47D) tumor cells were equally active. In addition, supplementation with estradiol did not modify the level of stimulation of collagen synthesis.

A stimulation of stromal cells proliferation by breast tumor matrix has been reported in the desmoplastic reactions [10]. In our culture conditions, this mechanism is unlikely to operate since mitomycin-treated MCF7 cells cocultured with fibroblasts failed to stimulate the proliferation rate of the latter. Furthermore, thymidine incorporation in coculture was identical to the sum of incorporation by both cell populations. The enhanced collagen synthesis persisted in mitomycin-treated fibroblasts in cocultures. Together, these results indicate that the stimulating effect is independent of fibroblast proliferation.

The electrophoretic pattern of labeled collagen polypeptides was identical in monoculture of fibroblasts and in coculture with MCF7 cells. Procollagen as well as partially processed p-C and p-N collagens and fully processed collagen types I, III, and V were identified. Collagen type VI cannot be clearly visualized using this electrophoretic procedure. Using radioimmunological titration, we observed an increase of type I, III, and VI collagens and fibronectin but the relative proportion of these polypeptides was not modified by the presence of MCF7 cells. Types I, III, and V collagen [2] and fibronectin [3] are known to accumulate in breast carcinoma in vivo. No data concerning type VI collagen have yet been reported to our knowledge.

The biosynthesis of collagens can be regulated at a number of transcriptional and post-transcriptional steps. We observed that the stimulation of collagen types I and III as well as fibronectin was mediated by parallel variations in the steady-state level of their specific mRNAs. Several studies have already indicated a coordinated regulation of the expression of these two types of collagen and its control mainly at a pre-translational level [12,34]. On the contrary, the increased production of collagen type VI appeared regulated at a post-transcriptional level since no coordinate enhancement in the steady-state level of mRNAs coding for the three polypeptide chains of this collagen was observed. The concomitant stimulation of collagen type VI secretion in the medium demonstrated a net increase of this collagen production rather than a stimulation of the deposition of newly synthesized protein into the extracellular matrix. The regulation of the different chains of collagen type VI expression in cultures of fibroblasts has also been reported to differ from that of collagen type I and III by Hatamoshi et al. [34].

Stimulation of collagen production by fibro-

blasts could be reproduced using conditioned medium of competent tumor cells (MCF7 cells, T47D cells, SA52 cells) suggesting that it could be mediated by soluble factor(s). However, the extent of the stimulation observed in coculture was always higher than what can be achieved using CM. A similar observation was also reported for collagenase [49] or proteoglycans [7] stimulatory activity suggesting that the mechanism of exchange of information might depend on a cell-to-cell or a paracrine interaction rather than stability or continuity of production of soluble factors. The chemical nature of the stimulating factor(s) is presently under investigation. There is clearly no resemblance to the previously described cytokines and growth factors known for their activity on fibroblasts.

In conclusion, this report demonstrates that some breast adenocarcinoma cells can modulate the phenotype of fibroblasts without affecting their proliferative response. It supports the concept that extracellular matrix surrounding some tumors depends upon specific host-tumor cell interactions.

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