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# Participation of Tenascin and Transforming Growth Factor-β in Reciprocal Epithelial-Mesenchymal Interactions of MCF7 Cells and Fibroblasts

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## ABSTRACT

**The tumor stroma is essential for the development of the tumor epithelium. Tenascin is an extracellular matrix protein highly expressed in the stroma of malignant mammary tumors. We therefore tested whether *in vitro* MCF7 cells were able to induce fibroblasts to synthesize tenascin. Indeed MCF7 cell-conditioned medium contained tenascin-inducing activity. This activity was shown to be transforming growth factor-β. The morphology of the MCF7 cells was in turn affected by the addition of tenascin to the culture medium. The cells partially detached from the substratum and lost their cell-cell contacts.**

## INTRODUCTION

Cancer can be seen "as part of an inexorable process in which the organism falls behind in its ceaseless effort to maintain order" as it was stated by Rubin (1). There are many mechanisms postulated to cause the loss of order: The induction of oncogenes caused by the introduction of viral genes or by activation of certain cellular genes (2) and alternatively or in combination with it the loss of tumor suppressor genes (3, 4). Since cancer cells, however, do not exist isolated in an organism, but are surrounded by other cells, their fate is also influenced by the environment. For example, the stroma around tumors is different from the normal organ stroma and it is believed to be critically involved in malignant growth (5, 6).

We recently showed that the extracellular matrix protein tenascin is highly expressed in the stroma of malignant but not of benign mammary tumors (7). We demonstrated that the tumor epithelium induces its surrounding stroma to lay down a tenascin-containing matrix *in vivo* (8).

In the present study, we examined whether a mammary carcinoma cell line is able to secrete factors stimulating the expression of tenascin in fibroblasts *in vitro* and how the tumor cells in turn are affected by the presence of tenascin. We show that TGFβ[1] secreted by MCF7 cells induces the production of tenascin by fibroblasts and that the MCF7 cells react to exogenous tenascin by losing cell-cell and cell-substrate contacts.

## MATERIALS AND METHODS

**Epithelial Cell Cultures.** T47D and MCF7 human mammary carcinoma cells (American Type Culture Collection) were grown in Dulbecco's medium (GIBCO, Basel, Switzerland) containing antibiotics and 10% FCS (Flow, Baar, Switzerland). Conditioned medium of nearly confluent MCF7 cells was prepared as follows. The cultures were washed with medium without FCS three times by incubating the culture at 37°C for 30 min between washes. The conditioned medium was then collected after incubation of the culture in medium containing 0.3% FCS for 24 h. T47D cells were plated on fibronectin-coated wells in the presence or absence of 20 μg/ml of tenascin in serum-containing medium. The fibronectin was purified from horse serum (9) and the tenascin was isolated from chick fibroblast conditioned medium as described (10, 11). In some cases MCF7 cells were plated on top of collagen gels prepared from rat tail collagen (Sigma, St. Louis, MO) according to the procedure of Michalopoulos and Pitot (12).

**Induction of Tenascin in Fibroblast Cultures.** Chick fibroblast cultures were prepared by trypsinization of the skin of 11d chick embryos. The cells were plated in 96-well cluster plates (Falcon, Becton, Dickinson and Co., Cockeysville, MD) in Dulbecco's medium supplemented with antibiotics and 0.3% FCS at $5 \times 10^4$ cells/well. Two days after plating, these cultures were used to assay for the induction of tenascin secretion. Preparations to be tested were added to the cultures for 16 h before the conditioned medium was collected and assayed for the tenascin content by enzyme-linked immunosorbent assay as described (13).

A neutralizing antibody against TGFβ was purchased (RD Systems, Inc., Minneapolis, MN). Conditioned medium of MCF7 cells as well as control medium containing 0.3% FCS was incubated with the indicated amounts of anti-TGFβ for 30 min before adding the medium to fibroblast cultures. The results of tenascin induction were obtained by subtracting the control values from the values obtained for the MCF7-CM.

**Immunoprecipitation, Immunofluorescence, and Northern Blots.** Anti-tenascin antiserum (11), anti-fibronectin antiserum (14), monoclonal anti-tenascin anti-TnM1 (15), and monoclonal anti-fibronectin anti-FnM6 (13) have been described.

Immunoprecipitations from metabolically labeled cell cultures using 100 μCi/ml of [$^{35}$S]methionine (New England Nuclear, Zurich, Switzerland) were performed as described (11). They were either analyzed on fluorographed sodium dodecyl sulfate-polyacrylamide gels (11) or the radioactive gel bands were cut from the dried gels and dissolved in 0.5 ml of 1 N NaOH at 65°C for 6 h. The solution was neutralized with acetic acid and counted in 5 ml of Irgascint (Ciba Geigy, Basel, Switzerland) for quantitation.

Immunofluorescence of cell cultures was performed as described (16).

Isolation of RNA and northern blots were performed using the tenascin complementary DNA probe cTn8 described in Pearson *et al.* (13).

## RESULTS

**MCF7-conditioned Medium Induces Tenascin Synthesis in Fibroblasts.** Chick embryo fibroblasts are able to secrete large amounts of tenascin when cultured in 10% FCS (Fig. 1*A, lane c*). If the serum is reduced to 0.3% FCS the secretion of total proteins is not affected; however, the secretion of tenascin is drastically reduced (Fig. 1*A, lanes b* and *d*). It is therefore possible to regulate the secretion of tenascin by exogenous factors. We tested whether the MCF7 mammary tumor cell line was secreting factors stimulating the synthesis of tenascin in fibroblasts. The MCF7 cells themselves do not synthesize any detectable tenascin. Chick embryo fibroblasts were used as the indicator cells for inducing substances, since we have chick-specific monoclonal antibodies available to detect the cellular source of the protein as well as cDNA probes of chick tenascin to analyze mRNA levels. As shown in Fig. 1*B* by northern blot analysis, the MCF7-CM was able to induce the accumulation of tenascin mRNA to the same extent as the addition of 10% FCS.

The accumulation of tenascin mRNA was accompanied by increased secretion of tenascin into the culture medium. The

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[1] The abbreviations used are: TGFβ, transforming growth factor-β; FCS, fetal calf serum.

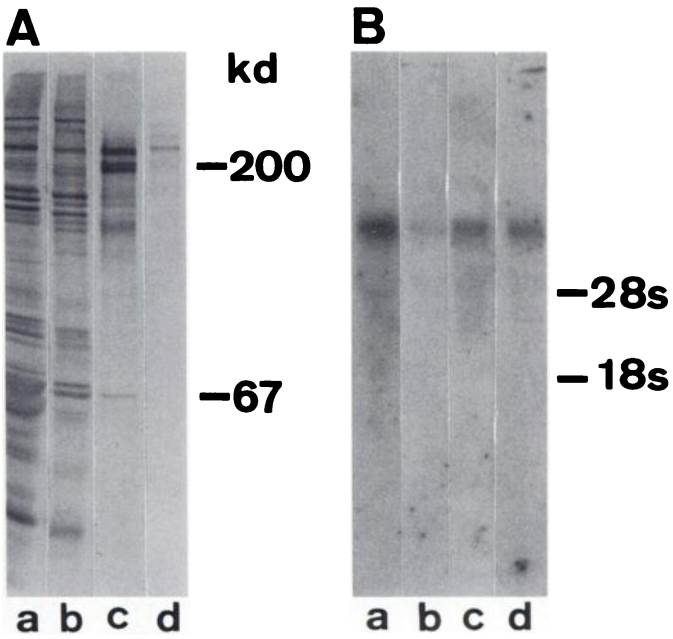


Fig. 1. Tenascin induction by FCS and MCF7-conditioned medium. *A*, conditioned media (10 μl/lane) of metabolically labeled chick fibroblasts grown in 10% FCS (*a*) or 0.3% FCS (*b*) and immunoprecipitations with antitenascin of 100 μl aliquots of these conditioned media from the cells in 10% FCS (*c*) or from 0.3% FCS (*d*). *B*, Northern blot hybridized with a chick tenascin complementary DNA of RNA (5 μg/lane) isolated from cells grown in 10% FCS (*a*), or 0.3% FCS (*b*) and parallel cultures in 0.3% FCS, which were switched to 10% FCS (*c*) or to MCF7-CM in 0.3% FCS (*d*) for 16 h before extraction of the RNA.

Table 1 *Stimulation of tenascin and fibronectin secretion by FCS and MCF7-conditioned media*

| Medium[a] | Addition[b] | Secretion of | |
|---|---|---|---|
| | | Tenascin | Fibronectin |
| 10% FCS | 10% FCS | 97% | 69% |
| 10% FCS | MCF7-CM (10% FCS) | 100% | 100% |
| 0.3% FCS | 0.3% FCS | 21% | 34% |
| 0.3% FCS | MCF7-CM (0.3% FCS) | 69% | 97% |
| 0.3% FCS | 10% FCS | 86% | 84% |

[a] Initial culture medium.
[b] Medium switch at 2 days of culture. Numbers were calculated from cpms counted in gel bands of immunoprecipitations of the conditioned media. The highest counts were taken as 100%. Maximal counts for fibronectin were about three times higher than maximal counts measured for tenascin.

results of one representative experiment are shown in Table 1. The secretion of tenascin was compared to fibronectin and quantitated by counting immunoprecipitated gel bands. The chick fibroblasts were grown in either 0.3% or 10% FCS and after 2 days they were switched to fresh medium with either concentration of FCS or to medium conditioned by MCF7 cells containing the corresponding concentration of FCS. At the time of the medium change [$^{35}$S]methionine was added and after 24 h the media were collected and analyzed. Tenascin as well as fibronectin secretion was stimulated by the addition of MCF7-CM. Their secretion was, however, differentially affected by FCS *versus* MCF7-CM. Whereas tenascin secretion was also maximally stimulated by the addition of FCS alone, fibronectin secretion was maximal only in the presence of MCF7-CM and was less affected by FCS. This differential accumulation of tenascin and fibronectin in the CM was consistently observed in all three experiments of this type performed.

**Tenascin-inducing Factor from MCF7 Cells is TGFβ.** It is known from the studies of Knabbe *et al.* (17) that MCF7 cells secrete biologically active TGFβ. Conversely, we know from our own research that tenascin synthesis is stimulated by TGFβ (13). We therefore tested whether anti-TGFβ was able to neutralize the tenascin-inducing activity produced by the MCF7 cells. As shown in Fig. 2, preincubation of the MCF7-CM with anti-TGFβ before adding it to fibroblast cultures neutralized its effect in a dose-dependent manner. Since at the highest dose of antibody the effect of the CM was completely abolished, TGFβ seems to be an indispensable factor required for the tenascin inducing activity found in the MCF7-CM.

**TGFβ and MCF7 Cells Promote Tenascin and Fibronectin Deposition in the Extracellular Matrix of Fibroblasts.** TGFβ not only promotes the secretion of tenascin and fibronectin, but it also stimulates their deposition in the extracellular matrix as shown in Fig. 3.

When MCF7 cells were plated on top of an established monolayer of fibroblasts, the fibroblasts surrounding the MCF7 cells started laying down a tenascin-rich extracellular matrix. The clusters of MCF7 cells are not stained by anti-tenascin antiserum. Since we were in addition using a chick-specific monoclonal anti-tenascin (not shown) the cellular source of tenascin was unquestionably from the fibroblasts and the MCF7 cells did not secrete any tenascin, but were able to induce the local production of a tenascin-rich matrix in the surrounding fibroblasts.

**Tenascin Disrupts Epithelial Cell Contacts of Mammary Tumor Cells.** The two human mammary carcinoma cell lines T47D and MCF7 were cultured on either fibronectin-coated dishes or on top of a collagen gel. Both cell lines attached to their substrates and formed epithelial sheets with tight cell-cell contacts making the boundaries between single cells difficult to discern (Fig. 4, *a* and *c*). When the T47D cells were plated in the presence of tenascin in the medium they did not attach to fibronectin and formed no tight cell-cell contacts (Fig. 4*b*). The MCF7 cells were plated on top of collagen gels without tenascin in the medium. Four days later tenascin was added to the full medium and within 2 days the epithelial sheets had retracted and numerous single cells had left the epithelial cell clusters (Fig. 4*d*).

## DISCUSSION

All tumors require stroma if they are to grow beyond a minimal size (18). Therefore the hope has been raised that

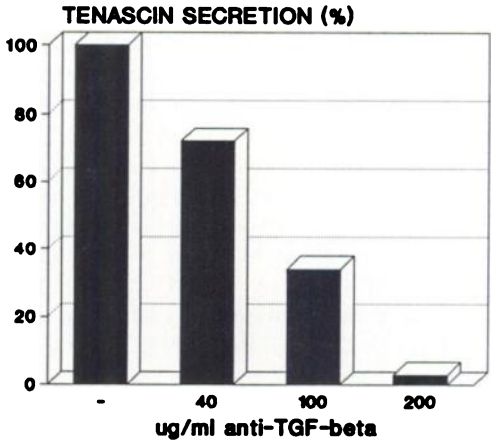


Fig. 2. Anti-TGFβ neutralizes the effect of the MCF7-CM. Tenascin secretion by chick fibroblasts in the presence of MCF7-CM was determined by enzyme-linked immunosorbent assay in the absence or presence of anti-TGFβ at the concentrations indicated. Tenascin secretion in MCF7-CM in the absence of anti-TGFβ was taken as 100%. *Bars*, average of four wells analyzed and none of the values measured differed more than 15% from each other. The addition of equal amounts of an unrelated rabbit IgG did not inhibit the activity of the MCF7-CM.

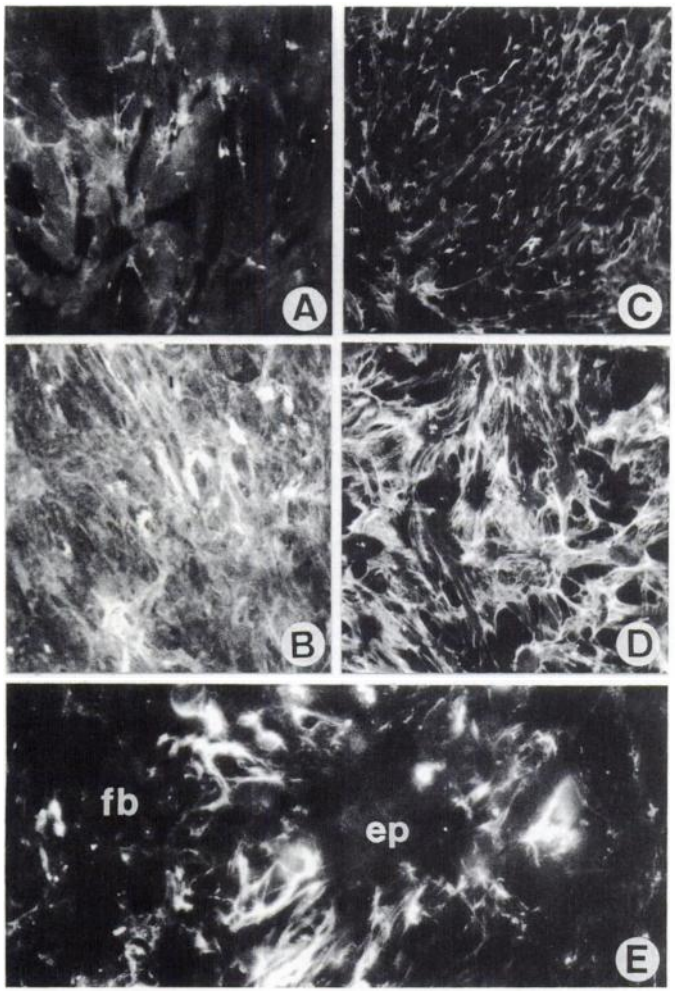


Fig. 3. TGFβ and MCF7 cells induce the desposition of a tenascin-rich matrix in fibroblasts. Chick fibroblasts were cultured for 2 days in 0.3% FCS in the absence (*A, C*) or the presence (*B, D*) of 20 ng/ml TGFβ. *A* and *B*, staining with antitenascin; *C* and *D* staining with anti-fibronectin. In *E*, MCF7 cells have been cultured for 2 days on top of a fibroblast monolayer in 0.3% FCS. The staining with antitenascin revealed the local production of a tenascin-rich matrix in the fibroblasts (*fb*) immediately surrounding the epithelial cells (*ep*).

approaches to cancer treatment may be directed at preventing the generation of tumor stroma (19). In addition there is experimental evidence that the stroma is actively involved in carcinogenesis (compare with Refs. 5, 6). In the light of these considerations it is certainly of relevance to study the factors which promote the formation of this tumor stroma. Several laboratories have found factors present in the conditioned medium or the extracellular matrix of tumor cell lines which stimulate the growth or the synthesis of extracellular matrix components by fibroblasts (20–24). However, none of these factors have been fully characterized. In the *in vitro* model system used in this study we were able to show that one such factor is TGFβ. TGFβ has been shown to be important for the regulation of cell differentiation and embryonic development (for reviews see Refs. 25, 26). Recently (27) it was shown that TGFβ mRNA was synthesized in the epithelia overlying the mesenchymes which previously had been shown to contain TGFβ protein (28). An analogous situation can be found in our *in vitro* test system, namely that the epithelial MCF7 cells are secreting TGFβ which is acting on the surrounding mesenchymal cells. Obviously a similar situation may be postulated for *in vivo* tumors.

We tested the inductive action on two extracellular matrix glycoproteins, fibronectin and tenascin. Both of them are believed to be important in regulating cell-substrate adhesion (29, 30). Whereas fibronectin favors adhesion and spreading of many different cell types, tenascin has the opposite effect and interferes with cell adhesion (31).

Previous studies showed that the stroma of malignant mammary tumors laid down a tenascin-containing matrix but not the stroma of benign tumors (7, 8). Since tenascin in contrast to fibronectin shows a much more restricted tissue distribution and is highly expressed in many types of malignant tumors (for review see Ref. 30), monoclonal anti-tenascin antibodies have been successfully used for treatment of tumors in human glioma xenograph models (32).

In the model system described here tenascin was able to promote the release of single cells from epithelial cell sheets *in vitro* and therefore could possibly also aid cells to leave an original tumor *in vivo* allowing them to invade the surrounding tissues and ultimately metastasize. The importance of cell-cell contact and cell shape in neoplasia has been discussed recently (33). In a study of human colon cancer cells, it was reported that the loss of actin organization appeared to mark the transition of noninvasive benign to invasive malignant tumor cells (34). One possibility for tenascin to actively contribute to the malignant behavior of a tumor could be that it causes the rounding up of cells by promoting the disintegration of the actin fibers.

In an earlier study we had shown, that a tenascin substrate promotes the growth of mammary tumor cells (11). The results described in this paper provide further evidence for the critical involvement of the extracellular matrix of the tumor stroma not only for the growth but also for the morphology and cellular adhesion of the tumor cells. It is therefore likely that the composition of the stromal extracellular matrix actively partic-

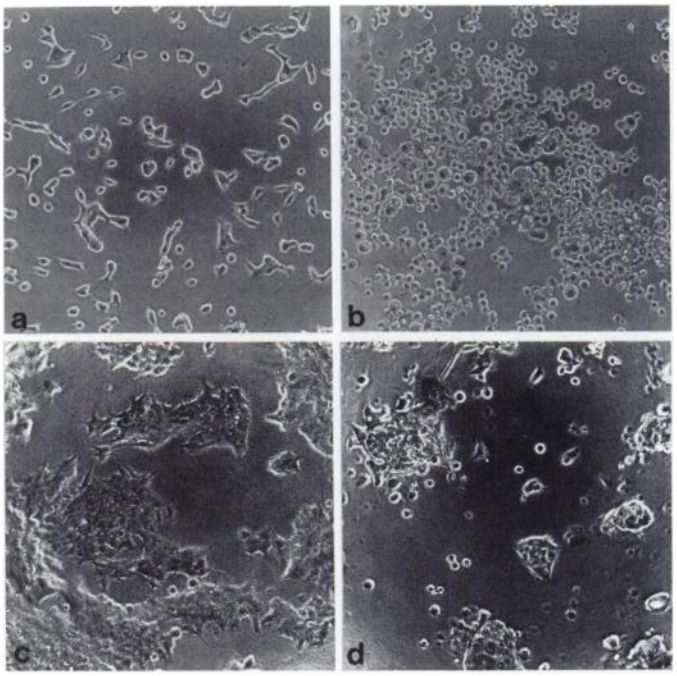


Fig. 4. Tenascin disrupts epithelial cell-cell contacts. T47D cells plated on fibronectin-coated wells without (*a*) or with (*b*) tenascin in the medium. The cells do not attach to the substrate nor do they form tight cell-cell contacts. MCF7 cells were grown for 4 days on top of a collagen gel. Then the medium was replaced by tenascin-free (*c*) or tenascin-containing (*d*) fresh medium and 2 days later the photographs shown were taken. In the presence of tenascin the epithelial sheets had contracted and numerous single cells became visible.

ipates in determining the growth and metastatic behavior of the cells in a tumor.

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