Tenascin-C signaling through induction of 14-3-3 tau

Doris Martin, Marianne Brown-Luedi, and Ruth Chiquet-Ehrismann

Friedrich Miescher Institute, Novartis Forschungsstiftung, CH-4002 Basel, Switzerland

We searched by a cDNA subtraction screen for differentially expressed transcripts in MCF-7 mammary carcinoma cells grown on tenascin-C versus fibronectin. On tenascin-C, cells had irregular shapes with many processes, whereas on fibronectin they were flat with a cobble stone–like appearance. We found elevated levels of 14-3-3 tau transcripts and protein in cells grown on tenascin-C. To investigate the consequences of an increased level of this phospho-serine/threonine–binding adaptor protein, we transfected MCF-7 cells with a construct encoding full-length 14-3-3 tau protein and selected clones with the highest expression levels. The morphology of these cells on tenascin-C was flat, resembling that of cells on fibronectin. This was reflected by a similar pattern of F-actin staining on either substratum. Furthermore, the growth rate on tenascin-C was increased compared with the parental cells. After transient transfection of HT1080 fibrosarcoma and T98G glioblastoma cells with 14-3-3 tau, only the 14-3-3 tau–expressing cells were able to adhere and survive on tenascin-C, whereas all cells adhered well on fibronectin. Therefore, we postulate that tenascin-C promotes the growth of tumor cells by causing an increase in the expression of 14-3-3 tau, which in turn has a positive effect on tumor cell adhesion and growth.

Introduction

Tenascin-C is an ECM protein well known for its high expression in almost all solid tumors and for its antiadhesive properties for cells in culture (for reviews see Chiquet-Ehrismann, 1993; Crossin, 1996; Vollmer, 1997; Jones and Jones, 2000; Orend and Chiquet-Ehrismann, 2000). The level of tenascin-C expression and the nature of the isoform expressed seems in a variety of tumors to correlate with a higher incidence of metastases and poor prognosis (Goepel et al., 2000; Emoto et al., 2001; Salmenkivi et al., 2001; Adams et al., 2002; Herold-Mende et al., 2002).

Therefore, it is of great interest to find out how tumor cells react to the presence of tenascin-C. In most of the published studies to date, effects of tenascin-C on cell adhesion, spreading, migration, and growth have been reported (for review see Orend and Chiquet-Ehrismann, 2000). However, the molecular mechanisms mediating these responses to tenascin-C remain largely unknown. From previous studies we know that a tenascin-C substratum supports the growth of rat primary mammary carcinoma cells more than fibronectin (Chiquet-Ehrismann et al., 1989). Furthermore, we have shown that MCF-7 human mammary carcinoma cells induce tenascin-C expression in cocultured fibroblasts surrounding the tumor cell nests (Chiquet-Ehrismann et al., 1989).

We therefore decided to screen for transcripts that are differentially expressed in MCF-7 cells grown on a tenascin-C versus a fibronectin substratum. We now report on our discovery that a tenascin-C substratum induces the expression of the phospho-serine/threonine–binding adaptor protein 14-3-3 tau in MCF-7 mammary carcinoma cells and describe its influence on cell adhesion and cell growth.

Results and discussion

MCF-7 cells were grown in medium containing 10% FCS for 24 h on plates coated with fibronectin or tenascin-C, respectively. The cells show completely different morphologies on these two substrates. They grow in epithelial patches on fibronectin but lose their cell–cell contacts and adopt irregular shapes on a tenascin-C substratum (Fig. 1). This is reminiscent of the results obtained with MCF-7 cells grown on collagen gels in the presence and absence of tenascin-C where the cells in the presence of tenascin-C were found to loose their cell–cell contacts and detached from the substratum (Chiquet-Ehrismann et al., 1989). To identify molecular differences between the cells grown on fibronectin versus tenascin-C, we isolated mRNA from these cells and performed a screen for differentially expressed transcripts. This screen resulted in the identification of a cDNA clone encoding the adaptor protein 14-3-3 tau. 14-3-3 proteins are a family of phospho-serine/threonine–binding proteins with >70 known ligands as diverse as kinases, phosphatases, receptors, structural proteins, and transcription...
factors (for reviews see Fu et al., 2000; van Hemert et al., 2001; Tzivion and Avruch, 2002). We confirmed the higher level of 14-3-3 tau transcripts in two mRNA batches isolated from independently cultured MCF-7 cells on tenascin-C versus fibronectin substrates by semiquantitative PCR. As shown in Fig. 2 A, transcript levels of GAPDH were equal, whereas 14-3-3 tau was highly increased in the samples from the cells cultured on tenascin-C compared with fibronectin.

We next tested whether the level of the 14-3-3 tau protein was also affected. We loaded equal amounts of cellular protein on an SDS-PAGE followed by detection of 14-3-3 tau by a specific antiserum on an immunoblot. As shown in Fig. 2 B, the level of the 14-3-3 protein was also much higher in cell extracts of cells grown on a tenascin-C substratum than on fibronectin.

From the literature we know that 14-3-3 proteins are implicated in the regulation of cell growth and oncogenic transformation (Takihara et al., 2000) and that they exhibit anti-apoptotic activity (Xing et al., 2000; Masters and Fu, 2001). Therefore, we decided to test for an effect of elevated 14-3-3 tau levels in MCF-7 cells on their adhesion and growth behavior when cultured on tenascin-C versus fibronectin substrates. We transfected MCF-7 cells with a construct encoding 14-3-3 tau containing an NH$_2$-terminal Flag tag. Cell clones were isolated and tested on immunoblots for expression of the transfected 14-3-3 tau in comparison to the endogenous protein as shown in Fig. 3 A. Clones 2 and 5 exhibiting the highest amount of the transfected 14-3-3 tau protein were selected for further experiments. When they were grown on tenascin-C–coated plates they exhibited a cobblestone-like morphology as on fibronectin substrates by semiquantitative PCR. As shown in Fig. 2 A, transcript levels of GAPDH were equal, whereas 14-3-3 tau was highly increased in the samples from the cells cultured on tenascin-C compared with fibronectin.

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fibronectin, whereas the parental cells showed an irregular morphology on tenascin-C (Fig. 3 B). This was particularly well visible in phalloidin-stained cells (Fig. 4 A).

Two more cell lines were analyzed for a similar effect of 14-3-3 tau on cell adhesion on tenascin-C. HT1080 fibrosarcoma cells and T98G glioblastoma cells were transiently transfected with 14-3-3 tau after plating them on either a fibronectin or a tenascin-C substratum. 1 d later, transfected cells were visualized by staining the Flag tag of the transfected 14-3-3 tau, and all cells were stained by phalloidin (Fig. 4 B). On fibronectin, a small proportion of the large number of adhering cells expressed 14-3-3 tau, whereas on tenascin-C only a few cells were present but all expressing 14-3-3 tau. This implies that in these cells overexpression of 14-3-3 tau also led to an increased cell adhesion on tenascin-C.

Possibly, this effect is related to the reported interaction of 14-3-3 tau with cell adhesion on tenascin-C. HT1080 fibrosarcoma cells and T98G glioblastoma cells were transiently transfected with 14-3-3 tau after plating them on either a fibronectin or a tenascin-C substratum. 1 d later, transfected cells were visualized by staining the Flag tag of the transfected 14-3-3 tau, and all cells were stained by phalloidin (Fig. 4 B). On fibronectin, a small proportion of the large number of adhering cells expressed 14-3-3 tau, whereas on tenascin-C only a few cells were present but all expressing 14-3-3 tau. This implies that in these cells overexpression of 14-3-3 tau also led to an increased cell adhesion on tenascin-C.

We next tested the effect of the overexpression of 14-3-3 tau on cell growth. We plated equal numbers of cells on fibronectin versus tenascin-C substrates in complete medium and cultured the parental versus the 14-3-3–transfected clones 2 and 5 for 3 d. After 3 d in culture, the MCF-7 cells reached higher densities on fibronectin than on tenascin-C, whereas no distinction could be made for clone 2 or 5, respectively (unpublished data). The lower cell number of the parental MCF-7 cells on a tenascin-C substratum coincided with a reduced level of DNA replication on a tenascin-C substrate in comparison to fibronectin as measured by $^{3}$H-thymidine incorporation (Fig. 5). The $^{3}$H-thymidine incorporation by the 14-3-3 tau–overexpressing clones 2 and 5 was recovered on tenascin-C and reached values almost as high as on fibronectin (Fig. 5). A possible mechanism is that cell cycle progression is stimulated by an interaction of 14-3-3 with phosphorylated p27$^{Kip1}$ and by retaining this cell cycle inhibitor in the cytoplasm (Fujita et al., 2002). Alternatively, stimulation of cell growth by 14-3-3 could be indirect by its known inhibitory action on apoptosis (Xing et al., 2000; Masters and Fu, 2001). To test whether cells on tenascin-C were protected from apoptosis by overexpression of 14-3-3 tau, we plated the parental MCF-7 cells and clones 2 and 5 on tenascin-C– and fibronectin-coated wells, respectively, and analyzed the number of apoptotic cells under

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Figure 4. Effect of 14-3-3 tau on cell morphology and adhesion on fibronectin versus tenascin-C substrata. (A) TRITC-phalloidin staining of MCF-7 parental cells and clones 2 and 5 overexpressing 14-3-3 tau grown in fibronectin-coated (FN) or tenascin-C–coated (TN) wells. MCF-7 cells are well spread on fibronectin but elongated with many actin-rich processes on tenascin-C as opposed to clones 2 and 5, which show similar morphologies on either substrate with actin accumulations at the cell peripheries of the well spread cells. Bar, 50 μm. (B) Anti-Flag staining reveals cells overexpressing transfected 14-3-3–Flag (14-3-3), and RITC-phalloidin staining shows F-actin in all cells present in the same field of cultures of HT1080 cells and T98G cells grown on either fibronectin (FN) or tenascin-C (TN). Only the transfected cells were able to adhere and survive on the tenascin-C substratum, whereas on fibronectin most cells remained present. Bar, 50 μm.
both conditions. However, not even the parental MCF-7 cells showed increased apoptosis on tenascin-C compared with the cells plated on fibronectin. Reduction of the serum level in the medium to 1% lead to a concomitant increase in apoptotic cells on both substrates. Therefore, the hypothesis that 14-3-3 tau could protect MCF-7 cells from apoptosis cannot be the reason for the observed increase in growth.

Clearly 14-3-3 proteins are important adaptor proteins with many target proteins such as Raf-1 (Fanti et al., 1994; Fu et al., 1994; Li et al., 1995), PKC (Van Der Hoeven et al., 2000), phosphatidylinositol 3-kinase (Bonneworf-Berard et al., 1995), Bad (Zha et al., 1996), and Cdc25 (Kumagai and Dunphy, 1999) and thus seem to have a central and integrating role in orchestrating signaling pathways regulating cell growth and death. Furthermore, their positive effect on cell adhesion presents an additional input in their capability of influencing cell behavior. For cells that have not completely lost their anchorage dependence, this again has a positive effect on cell growth. Therefore, it is interesting that tenasin-C, an extracellular matrix protein highly overexpressed in cancer tissues, causes an increase in the expression of 14-3-3 tau in cancer cells. Tenasin-C may, therefore, alter the physiologic status of the tumor cells by induction of 14-3-3 tau, thereby promoting their growth.

Materials and methods

Cell cultures

MCF-7 mammary carcinoma cells (HTB-22; American Type Culture Collection), HT1080 fibrosarcoma cells (CCL-121; American Type Culture Collection), and T98G glioblastoma cells (CRL-1690; American Type Culture Collection) were cultured in Dulbecco’s medium (Life Technologies) containing 10% FCS (Amimed) at 37°C and 5% CO₂. Substrates of tenasin-C or fibronectin were prepared by coating these proteins at 25 μg/ml in PBS including 0.01% Tween for 1 h at RT. Tenasin-C and fibronectin were purified as described previously (Fischer et al., 1997).

Cell growth was analyzed by measuring the incorporation of ³H-thymidine. Equal numbers of cells were plated on tenasin-C versus fibronectin in complete medium. After 22 h, the medium was removed, the cell layers were washed with medium without FCS, and medium containing 0.1% FCS was added. 22 h later, the cells were labeled with ³H-thymidine for 6 h and harvested and analyzed as described previously (Huang et al., 2001). Apoptosis was analyzed by TUNEL staining using the In Situ Cell Death Detection kit (Roche Diagnostics AG) according to the procedure of the manufacturer.

For immunofluorescence and F-actin staining, cells were fixed with 4% PFA in PBS for 30 min and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Then, they were either incubated for 1 h with 0.05 μM TRITC-phalloidin (Sigma-Aldrich) or with anti-Flag (M2; Stratagene) at a 1:500 dilution, or with both simultaneously, all in PBS containing 3% goat serum for 1 h. Secondary goat anti-mouse Alexa 488-conjugated antibodies (Molecular Probes) diluted 1:1,000 in PBS/3% goat serum were applied for detection of the primary antibodies. Cells were washed in PBS after each incubation.

Finally, the specimens were mounted in Mowiol (Calbiochem) and examined and photographed using an Axioshot microscope (Carl Zeiss Microimaging, Inc.) connected to a JCCD camera (Sony).

Subtractive cDNA cloning

An Oligoext Direct mRNA kit (QIAGEN) was used to isolate mRNA from two 10-cm plates of MCF-7 cells grown on either fibronectin or tenasin-C substrata for 24 h in complete medium. These mRNA samples were used for RT-PCR reactions and subtractive hybridization using the Advantage cDNA PCR kit (CLONETECH Laboratories, Inc.) and the PCR-Select cDNA Subtraction kit (CLONETECH Laboratories, Inc.) according to the manuals supplied. The PCR products were cloned into pKS and analyzed by sequencing. Primers were derived from the insert sequences to verify a differential expression of the respective transcripts in the two cDNA batches by semiquantitative PCR reactions using Taq polymerase (Roche Diagnostics AG). To amplify the 14-3-3 tau, we used the following primer pair: 5′-AGTTCGTCGCTGTGGTGATGATCG-3′ and 5′-ATGATGACCGCTTCGTA-3′. Samples were taken from each reaction after completion of 15, 20, 25, and 30 cycles and analyzed on agarose gels after ethidium bromide staining. This allowed us to verify the linear range of amplification that we used for comparison.

Transfection of 14-3-3–Flag and Western blots

A full-length 14-3-3 tau construct containing an NH₂-terminal Flag tag was engineered by RT-PCR from mRNA of MCF-7 cells using the Advantage cDNA PCR kit (CLONETECH Laboratories, Inc.). A first set of 14-3-3-specific primers was used (5′-GCGCGCGCCGAGGATGAC-3′ and 5′-AAGGATGACACCCTGTATGG-3′) followed by a second round of PCR with (5′-ACTGCCGCGCACTGACTACAAGGATGACGATGACAAGTGAGAAAGCTGAGTGCT-3′ and 5′-ATCTCCGAGTTATGGTCGCCCCTTTCTGC-3′) to add the Flag tag and the appropriate restriction sites NotI and XhoI at respective ends needed for subsequent cloning into the expression vector pcDNA3 (Invitrogen). The resulting construct was verified by sequencing and was called 14-3-3–Flag. It contained the complete coding sequence of human 14-3-3 tau available from GenBank/EMBL/DDJB under accession no. X56468 with an NH₂-terminal extension of a Flag tag. MCF-7 cells were transfected with 14-3-3–Flag using the transfection reagent fuge (Roche Diagnostics AG), and clones were selected for by the addition of G418 (Life Technologies). The resistant cell clones were tested for the production of the 14-3-3–Flag protein on Western blots of cell extracts using anti-Flag (M2; Stratagene), peroxidase-labeled anti-mouse IgG (Cappel/ICN), and the ECL reagent (Amersham Biosciences) for chemiluminescent detection of the reactive bands. Endogenous synthesis of 14-3-3 tau was detected on the same blots using anti-14-3-3 theta/tau (Research Diagnostics Inc.). The Flag-tagged 14-3-3 migrate slightly slower than the endogenous protein due to the presence of the tag.

HT1080 and T98G cells were each plated on fibronectin and tenasin-C–coated plates. 1 d later, they were transiently transfected with the 14-3-3–Flag construct using the transfection reagent fuge. On the next day, cells were fixed and stained with anti-Flag and phallolidin as described above for the MCF-7 cells.

We thank Wentao Huang for his expert help with the ³H-thymidine incorporation assays.

Submitted: 26 June 2002
Revised: 10 December 2002
Accepted: 10 December 2002

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