Suppression of Transformed Phenotypes of Human Fibrosarcoma Cells by Overexpression of Recombinant Fibronectin

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ABSTRACT

Loss of fibronectin (FN) from the cell surface has been shown to be closely associated with malignant transformation of cells. To elucidate the role of the FN matrix in the modulation of malignant phenotypes, we overexpressed full-length cDNA encoding plasma-type FN in HT1080 human fibrosarcoma cells. The cells overexpressing FN adopted a more flattened morphology and deposited a moderately developed FN matrix both in vitro and in vivo, although the level of expression of integrin α5β1 remained unchanged. FN-overexpressing cells exhibited a reduced cell motility on the substratum and grew poorly when injected s.c. into nude mice. Overexpression of FN also suppressed the ability of the tumor cells to proliferate in soft agar, whereas the suppression was reversed by inclusion in soft agar of the Arg-Gly-Asp (RGD)-containing peptide and adhesion-blocking antibodies against the central cell-binding domain of FN. Neither cell motility nor growth potential was altered by overexpression of a truncated form of FN lacking the central cell-binding domain. These results, taken together, indicate that increased deposition of FN in the pericellular matrix per se can suppress the motility and growth potential of tumor cells through interaction with RGD-recognizing integrins, most likely α5β1.

INTRODUCTION

FN is an extracellular matrix glycoprotein that plays important roles in many fundamental biological processes, including cell adhesion, proliferation, differentiation, cytoskeletal organization, and apoptosis (1). FN is synthesized by many cell types as disulfide-bonded dimers of closely related polypeptides that arise from a single RNA transcript by alternative splicing. The level of expression and the proportion of each spliced isoform change during embryonic development and in pathological processes including wound healing, inflammation, fibrosis, and oncogenic transformation (2–6).

A major source of early interest in FN had been the finding that FN on the cell surface disappears upon oncogenic transformation (7, 8). There is a good correlation between the loss of FN and acquisition of the malignant phenotype in vitro and of tumorigenic and metastatic phenotypes in vivo (9, 10). Conversely, reversion of transformed cells to the normal phenotype by cyclic AMP derivatives and butyrate was associated with reappearance of the pericellular FN matrix (11, 12). Introduction of the tumor suppressor gene Krev-1 into K-ras-transformed cells restored more flattened morphology along with reduced growth potential and increased expression of FN (13).

Despite the close association of the decrease in the cell surface FN with oncogenic transformation, the role of surface FN in the malignant growth of tumor cells remains poorly understood. It was previously shown that readdition of purified FN to virally transformed cells promoted cell adhesion to the substratum and reorganization of the microfilamentous cytoskeleton, thus restoring a normal morphology, but did not affect growth behavior of the tumor cells (14). On the other hand, elevated expression of integrin α5β1, the major receptor for FN that recognizes the RGD motif within the central cell-binding domain of FN, suppressed the growth potential of hamster tumor cells both in vitro and in vivo with increased deposition of FN on the cell surface (15).

During the past few years, a wealth of evidence has accumulated which establishes that adhesion to extracellular matrix proteins, particularly FN, through the integrin family of adhesion receptors transduces biochemical signals inside the cells that regulate cell proliferation, differentiation, and apoptosis (16, 17). The interaction of integrin α5β1 with FN promotes cell cycle progression through a cyclin-dependent pathway (18), and supports survival of cells through up-regulation of Bcl-2 expression (19). These observations prompted us to reinvestigate the biological consequences of the reduced expression of FN in tumor cells. In the present study, we overexpressed intact and truncated forms of recombinant FNs in HT1080 human fibrosarcoma cells to examine the effects of the reassembled FN matrix on migratory and proliferative potentials of the tumor cells. Our results showed that overexpression of FN restored more flattened morphology, reduced cell migration on the substratum, and suppressed tumor growth in vivo in an RGD-dependent manner.

MATERIALS AND METHODS

cDNA Construction. Two expression vectors were constructed: one for expression of intact plasma-type FN and the other for expression of a truncated form of FN consisting of the N-terminal 70-kDa and C-terminal 37-kDa regions. The latter recombinant protein has been designated miniFN. Construction of the miniFN expression vector was reported previously (20).

A cDNA encoding the full-length plasma-type FN was assembled from three cDNA fragments encoding: (a) the 5′ untranslated region through Val527; (b) Asp278 through Ile1450; and (c) Arg1451 through the 3′ untranslated region. The amino acids were numbered from the N-terminal pyroglutamic acid in the mature cellular-type FN (21). The first cDNA fragment encoding the 5′ untranslated region through Val527 was prepared as follows. A 1455-nucleotide HindIII-NcoI fragment excised from pAI70F2 (22) was inserted into HindIII/NcoI fragment excised from pAI70F2 (22) was inserted into HindIII/NcoI-cleaved pAI70DS (23), and then a 1737-nucleotide HindIII-Sall fragment encoding the 5′ untranslated region through the signal sequence derived from human factor C inhibitor, and Gin5-Val645 was excised from the resulting plasmid. The second cDNA fragment encoding Asp278-Ile1450 was prepared as follows. A 354-nucleotide Sall-AscI fragment excising Asp278-Val450 was excised from the miniFN expression vector pMTX1 (20). A cDNA clone encoding Tyr266-His53 was amplified from the total RNA extracted from the human fibroblast WI-38 cells by reverse transcription-PCR and obtained as a 917-nucleotide Accl-NcoI fragment. These two cDNA fragments were ligated to SalI/BamHI-cleaved pBluescript along with a 1223-nucleotide NcoI-BamHI fragment prepared from pFH154 (24) in tandem. The resulting plasmid was digested with SalI and BamHI to yield a 2494-nucleotide fragment encoding Asp278-Ile1450. The third cDNA fragment encoding Arg1451 through the 3′ untranslated region was prepared by ligation of a 1241-nucleotide BamHI-SalI fragment excised from pFH154 and a 2123-nucleotide SacI-HindIII fragment prepared from pFH154 and a 2123-nucleotide SacI-HindIII fragment.
excised from pFH1 (24) into BamHI/HindIII-cleaved pBluescript, and then excised as a 2950-nucleotide BamHI-NspV fragment.

For construction of a full-length FN cDNA, the second and the third cDNA fragments were cloned into SalI/NspV-cleaved pMTX1. The resulting plasmid was cleaved with HindIII and SalI and the first cDNA fragment, i.e., the 1737-nucleotide HindIII-SalI fragment, was ligated into these sites to yield an expression vector, pAIPFN, for expression of the full-length human plasma-type FN cDNA.

Cell Culture. Human fibrosarcoma HT1080 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Cells were maintained in DMEM supplemented with 10% FBS. For immunoblotting and immunofluorescence analyses, cells were grown in DMEM containing 10% FN-depleted FBS. FN-depleted FBS was prepared by passage through a gelatin-Sepharose column twice.

cDNA Transfection. Each expression plasmid was cotransfected into HT1080 cells along with a selection marker, pKcone, using the calcium phosphate precipitation method. G418-resistant colonies were randomly picked and expanded in 24-well culture dishes, then assayed for recombinant proteins secreted into the culture medium by dot blot analysis as described previously (23).

Immunoblotting. Approximately 1 × 10^6 cells were grown in DMEM containing FN-depleted 10% FBS for 2 days. The conditioned medium was collected and clarified by centrifugation at 10,000 × g. Aliquots of the supernatants were subjected to SDS-PAGE using 6% polyacrylamide gels under reducing conditions followed by electrophoretic transfer of proteins onto nitrocellulose membranes. The recombinant FN and miniFN were detected with the monoclonal anti-human FN antibody 136H using enhanced chemiluminescence detection reagents (Amersham, Buckinghamshire, United Kingdom).

Indirect Immunofluorescence. Cells were grown to confluence for 4 days in DMEM containing 10% FN-depleted FBS on Lab-Tek chamber slides (Nunc, Naperville, IL). The confluent cells were washed twice with PBS and blocked with 2% BSA for 15 min, then incubated with polyclonal anti-FN antibodies for 45 min at room temperature. After washing with PBS three times, the cells were incubated with rhodamine-conjugated goat anti-rabbit IgG antibody for 30 min at room temperature. The cells were washed three times and examined with an Olympus IMT-2 microscope. Photomicrographs were taken on Kodak Tri-X film.

Migration Assay. Cell migration was measured by an in vitro wound assay (25). Cells were plated in DMEM containing 10% FBS on 24-well plates. Initial plating was adjusted to yield subconfluent monolayers of the same cell density after 24 h. The monolayers were then wounded by scratching 1-mm lines with a plastic scraper. After washing twice with serum-free DMEM, the cells were incubated with rhodamine-conjugated goat anti-rabbit IgG (Zymed Laboratories, San Francisco, CA) after fixation in cold absolute acetone.

RESULTS

Overexpression of FN in HT1080 Cells. A full-length cDNA-encoding human plasma-type FN was transfected into the human fibrosarcoma cell line HT1080, and two independent clones stably overexpressing FN, designated FN/A24 and FN/B22, were isolated. Immunoblot analysis of the conditioned medium of these clones demonstrated that FN/A24 and FN/B22 cells secreted approximately 8-fold and 4-fold more FN, respectively, than untransfected HT1080 cells (Fig. 1). We also transfected HT1080 cells with a cDNA encoding a truncated form of FN, designated miniFN, consisting of the N-terminal 70-kDa region and the C-terminal 37-kDa region (20). miniFN lacks the central cell-binding domain containing the cell-adhesive RGD motif, but retains the ability to assemble into the extracellular matrix (20, 22, 23). A stable transfectant, designated miniFN/C1, secretion about seven times more miniFN than endogenous FN on the molar basis was isolated and used as a reference.

Morphology of the FN-overexpressing cells was significantly different from that of the parental cells. FN/A24 cells were more adherent to the substratum and displayed more flattened morphology with reduced cell-cell overlapping than untransfected cells (Fig. 2). The cells overexpressing miniFN did not show significant morphological alterations.

Increased expression of FN allowed HT1080 cells to assemble the FN matrix on the cell surface. Although the FN matrix was not detectable by immunofluorescence on untransfected cells, FN/A24 cells displayed a moderately well-developed FN matrix on the cell surface (Fig. 3). miniFN/C1 cells were also positive for FN immunofluorescence, but to a lesser extent than FN/A24 cells.

The increased assembly of the FN matrix on FN/A24 cells was not...
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Fig. 2. Morphology of HT1080 cells overexpressing recombinant FNs. Untransfected HT1080 cells (control HT) and those overexpressing either recombinant FN (FN/A24) or miniFN (miniFN/C1) were seeded at 1 × 10^6 cells/10-cm dish and grown in DMEM containing FN-depleted FBS for 2 days. Phase-contrast photomicrographs of the cells were taken with an Olympus IMT-2 microscope. Bar, 50 μm.

due to the increased expression of integrin α5β1, the major FN receptor, or integrin αvβ3 that also binds to the RGD motif of FN, since the levels of expression of integrin α5 and αv subunits were unchanged after transfection with FN cDNA (data not shown).

Suppression of Tumor Cell Migration. The interaction with the FN matrix is considered to be important for cell migration. The effect of FN overexpression on tumor cell migration was assessed by an in vitro “wound” assay (Fig. 4). FN/A24 cells were less migratory than untransfected cells, as the number of cells migrating into the wound space was 40% less than that of untransfected cells. A similar but slightly less pronounced decrease in the migratory ability was observed with FN/B22 cells. In contrast, no significant difference was observed between miniFN/C1 and untransfected cells, suggesting that the central cell-binding domain is involved in the suppression of tumor cell migration.

Suppression of Tumorigenicity. To examine the effects of FN overexpression on the growth potential of HT1080 cells, FN/A24 and control cells (i.e., untransfected cells and miniFN/C1 cells) were injected into nude mice s.c., and the growth of the tumors was followed over a period of 1 month. FN/A24 cells grew poorly and formed only small nodules when compared with untransfected cells (Fig. 5A). Suppression of in vivo tumor growth appeared to be dependent on the levels of FN expression, since FN/B22 cells that expressed approximately 50% less FN than FN/A24 cells produced tumors which were intermediate in size between FN/A24 and untransfected cells (Fig. 5B). No significant difference in the tumorigenic ability was detected between untransfected and miniFN/C1 cells, indicating that the signals transduced from the central cell-binding domain are involved in the suppression of in vivo tumor growth. Immunohistological examination confirmed deposition of FN matrices in the tumors produced by FN/A24 cells, although only a little, if any, FN matrix was detectable in the tumors produced by untransfected cells (Fig. 6).

Effect on in Vitro Proliferation. In accordance with the reduced tumorigenicity, FN-overexpressing cells grew at a slower rate under the standard in vitro culture conditions, i.e., in the presence of 10% FBS, than untransfected cells, although no difference in the growth rate was observed between untransfected and miniFN-transfected cells (Fig. 7). The reduction in growth rate was more evident for FN/A24 cells than for FN/B22 cells, being consistent with tumor growth in vivo.

Suppression of Growth in Soft Agar. The ability to grow in soft agar has been shown to be closely correlated with tumorigenicity in vivo. Since FN-overexpressing cells had reduced growth potential both in vivo and in vitro, their ability to form colonies in soft agar was compared with those of untransfected and miniFN-transfected cells. FN/A24 cells displayed a reduced ability to proliferate in soft agar, yielding about 50% less colonies than untransfected cells (Fig. 8). A similar reduction in colony-forming efficiency was observed with FN/B22 cells (data not shown). In contrast, no significant difference was observed between untransfected and miniFN-transfected cells, again indicating the involvement of the central cell-binding domain.

Fig. 3. Indirect immunofluorescence analysis of recombinant FNs on the cell surface. Cells were grown to confluence for 4 days in DMEM containing 10% FN-depleted FBS. The cells were labeled with affinity-purified polyclonal rabbit antibody against human FN followed by rhodamine-conjugated goat anti-rabbit IgG antibody. Photomicrographs were taken on Kodak Tri-X film with an Olympus IMT-2 microscope. Bar, 50 μm.

control HT  FN/A24  miniFN/C1
To confirm the role of the central cell-binding domain in the suppression of tumor cell growth in soft agar, control and FN-overexpressing cells were seeded in soft agar containing the GRGDSP peptide. Inclusion of GRGDSP, but not inactive GRGESP peptide (data not shown), was found to restore the colony-forming efficiency of FN/A24 cells to a level close to that of untransfected cells (Fig. 8). Furthermore, monoclonal antibodies that block the cell-adhesive activity of the central cell-binding domain enhanced the colony formation of FN/A24 cells when included in the soft agar medium, confirming the importance of the RGD motif within the central cell-binding domain in the suppression of anchorage-independent growth of the tumor cells by FN overexpression.

**DISCUSSION**

Despite the close correlation between the decrease in cell-surface FN and acquisition of oncogenic phenotype, it has not yet been determined whether the reduced FN expression is simply a result of oncogenic transformation, or whether it is actively involved in the acquisition of tumorigenicity and invasiveness. One straightforward approach to answer this question is to restore the FN matrix around transformed cells by cDNA transfection and investigate how the tumorigenic and invasive properties are affected. Our results showed that overexpression of recombinant plasma-type FN in human fibrosarcoma cells restored the pericellular FN matrix, induced the tumor cells to adopt a more flattened morphology but to be less migratory on the substratum, reduced the ability to grow in soft agar, and strongly suppressed tumor growth in vivo. These results provide gain of function evidence for the direct involvement of the FN matrix in the growth control of tumor cells, and indicate that the loss of the FN matrix from the tumor cell surface is not only involved in morphological alterations and reduced cell to substratum adhesiveness but also confers an advantage for certain types of tumor cells to acquire tumorigenic and invasive phenotypes. The phenotypic alterations we observed should not be due to clonal variation associated with selection of stable transfectants, since both clones selected for the high level expression of recombinant FN showed similar phenotypes with reduced migratory and tumorigenic potentials. In a separate transfection experiment, we obtained another clone overexpressing recombinant FN at a similar level and confirmed that it also showed a significantly reduced tumorigenic potential. In contrast, none of the transfectants overexpressing miniFN showed reduced cell migration and tumorigenicity.

The reduced growth potential of the FN-overexpressing cells in soft agar and in vivo is considered to be dependent on the interaction of the central cell-binding domain of FN with the FN receptor on the tumor cell surface, most likely integrin α5β1, since the transfectants overexpressing miniFN lacking the central cell-binding domain did not show any significant reduction in their growth potential. The importance of the central cell-binding domain was further supported by the

Fig. 5. *In vivo* tumor growth of HT1080 cells overexpressing recombinant FNs. Four-week-old BALB/c-nu/nu mice were given s.c. injections of 2 x 10⁶ untransfected HT1080 cells or those overexpressing recombinant FN or miniFN. The maximum and minimum diameters of the resulting tumors were measured over the skin twice a week to estimate the tumor volume. In A, tumor growth of clones FN/A24 (●) and miniFN/C1 (△) was compared with that of untransfected HT1080 cells (○). In B, tumor growth of two HT1080 clones overexpressing different levels of recombinant FN, FN/A24 (●), and FN/B22 (■) was compared along with untransfected HT1080 cells (○). Bars, SD.

4 R. Manabe, unpublished observation.
observation that the reduced colony formation of FN-overexpressing cells in soft agar was reversed to a level close to that of untransfected cells by blocking the cell-adhesive activity of the central cell-binding domain with monoclonal antibodies or GRGDSP peptide.

There is accumulating evidence that integrins transduce signals across the plasma membrane via a variety of pathways including Ca²⁺ influx, H⁺ exchange, protein tyrosine and nontyrosine phosphorylation, alterations in phosphoinositide metabolism, and activation of mitogen-activated protein kinase cascades, thereby regulating proliferation, differentiation, migration, and apoptosis of the cells (16, 26).

Binding of integrin α5β1 to substrate-coated FN has been shown to induce a hierarchy of transmembrane events leading to supramolecular assembly of cytoskeletal components and many kinds of signaling molecules at adhesion sites with concomitant activation of both mitogen-activated protein kinase and JNK (Jun kinase) pathways (17) and to prevent apoptosis of the cells, possibly through up-regulation of Bcl-2 expression (19, 27). In tumor cells, however, several lines of evidence indicate that the integrin α5β1 negatively regulates cell proliferation. Thus, variants of Chinese hamster ovary cells deficient in or with reduced levels of integrin α5β1 showed increased tumorigenicity (28), whereas overexpression of integrin α5β1 resulted in a significant decrease in anchorage-independent growth and tumorigenic potential (15, 29). Our results are in line with these observations and provide evidence that interaction of integrin α5β1 with FN transduces signals that suppress anchorage-independent tumor cell growth in soft agar and tumor growth in vivo. It should be also noted that restoration of the FN matrix per se is sufficient for the suppression of tumorigenicity and anchorage-independent tumor cell growth without up-regulation of integrin α5β1, provided that the tumor cells, like HT1080 cells, express an adequate number of the integrin molecule on their surface. In support of this notion, Steel and Harris (30) reported that repression of FN expression by FN antisense RNA led to increased tumorigenicity of mouse melanoma-normal fibroblast hybrid cells.

Recently, Varner et al. (29) reported that de novo expression of integrin α5β1 in α5β1-deficient colon carcinoma cells by cDNA transfection resulted in significant suppression of tumor cell growth both in vitro and in vivo through induction of growth arrest-specific gene 1 (gas-1), a gene known to induce cellular quiescence (31). Interestingly, ligation of α5β1 on these cells through attachment to a FN-coated substrate inhibited the induction of the gas-1 gene and activated transcription of immediate early genes such as c-fos, c-jun, and junB, indicating that the signals transduced by integrin α5β1 upon binding to FN do not suppress but rather stimulate the in vitro growth of the tumor cells. The reason for the apparent discrepancy between this and other observations on the nature of α5β1-mediated signals is not clear, but the cells plated on the substratum and those denied adhesion to the substratum or inoculated in vivo may well respond differently to the signals transduced by ligand-occupied integrin α5β1. Given that cell proliferation is regulated by intricate cross-talk of multiple signals from the cell surface receptors for growth factors/ cytokines and those from adhesion receptors like integrins, it is possible that different cell types respond differently to the signals transduced by ligand-occupied α5β1. In support of this notion, Wang et al. (32) reported that binding of integrin α5β1 to substrate-adsorbed FN did not stimulate but rather inhibited the DNA synthesis of HT1080 cells. Similar contradictory results were obtained for the modulation of apoptosis by integrin-mediated signals. Interaction of integrin α5β1 with FN has been shown to prevent apoptosis of endothelial cells (27) and some tumor cells (19), although it induced apoptosis in leukemia cells (33).
It should be noted that the recombinant FN overexpressed in this study was the plasma-type FN that lacked both alternatively spliced extra domains termed ED-A and ED-B. Previously, we showed that expression of the FN isoforms containing these extra domains, collectively referred to as "cellular-type FN," was elevated in fetal and tumor tissues, whereas those lacking these extra domains were predominantly expressed in normal adult tissues (5, 34). Although no clear differences have been found between plasma- and cellular-type FNs in their abilities to bind integrin α5β1, collagens, and heparin/heparan sulfate, it will be interesting to examine whether overexpression of cellular-type FNs equally suppresses tumor cell proliferation in vivo and in soft agar. Regulated expression of recombinant FNs with or without the extra domains in tumor cells may provide clues for better understanding the physiological significance of the oncodevelopmental modulation of molecular heterogeneity of FNs.

REFERENCES

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