Source of Oncofetal ED-B-containing Fibronectin: Implications of Production by Both Tumor and Endothelial Cells

Marta Midulla, Rakesh Verma, Massimo Pignatelli, Mary A. Ritter, Nigel S. Courtenay-Luck, and Andrew J. T. George

ABSTRACT

ED-B fibronectin (FN) is a FN isoform derived from alternative splicing of the primary transcript of a single gene. Its expression on tumor stroma and neoformed tumor vasculature and its absence, with few exceptions, in normal adult tissues imply a prognostic and diagnostic value for ED-B FN. We investigated the location and source of ED-B FN because this will be of importance both in understanding its role in tumor development and in designing strategies to target this molecule. We have confirmed that ED-B FN is expressed in the majority of breast and colorectal carcinoma tissue samples, with strong immunohistochemical staining around the tumor cells and in the tumor stroma. No staining of tumor neovasculature was seen. ED-B FN is produced by a range of tumor and endothelial (both primary and transformed) cell lines, as detected by reverse transcription-PCR, but is not expressed at the plasma membrane. Strong expression of human ED-B FN is seen in tumor xenographs. These data indicate that neoplastic cells can act as the source of ED-B FN in tumors. The lack of cell surface expression on tumor cell lines has clear implications for the design of therapeutic strategies which target this molecule.

INTRODUCTION

FN is a large adhesive glycoprotein and a normal constituent of extracellular fluids, extracellular matrices, most basement membranes, and many cell types. It is implicated in a variety of different biological phenomena such as cell adhesion, establishment and maintenance of normal cell morphology, cell migration, differentiation, transformation, hemostasis, thrombosis, wound healing, oncogenic transformation, and oncoproteins (1, 2).

Alternative splicing of the primary transcript of a single gene located on human chromosome 2, generates numerous distinct fibronectin transcripts that account for the individual α and β chains and for the plasma and cellular isoforms. Fibronectin polymorphism is due to alternative splicing of a 50-kb gene, which contains 50 exons, in three different regions, IIICS, ED-A, and ED-B of the primary transcript, as well as posttranslational modifications (3). Alternative splicing generates 20 different isoforms that differ in the number of internal repeats (3–10).

The alternative splicing of fibronectin pre-mRNA is regulated in a cell-, tissue-, and developmentally specific manner; for example, exon skipping by hepatocytes yields a fibronectin polypeptide that is missing an amino acid sequence that is present in cellular but not in plasma fibronectin. It has been demonstrated that the splicing pattern of FN mRNA is deregulated in transformed cells and in malignancies (3, 11–16).

Different fibronectin isoforms containing IIICS, ED-A, and ED-B repeats are expressed to a greater degree in tumor tissues and transformed human cell lines than in their normal counterparts (17). ED-B-containing fibronectin, with very few exceptions (superficial synovial cells, intima of some vessels and areas of interstitium of ovary, functional layer of endometrium during the proliferative phase, and isolated areas of basement membrane of celiac epithelium) is reported to be absent in normal adult tissues (3).

The ED-B domain is encoded by a single exon (complete type III homology) and is composed of 91 amino acids. Existing data indicate that ED-B fibronectin is associated with oncofetal fibroblasts. This suggests a prognostic and diagnostic value of ED-B fibronectin in tumors. Expression of different isoforms of fibronectin has been demonstrated in malignant and other disease conditions. It has been suggested that expression of these isoforms is correlated with remodeling of tissue during wound healing or embryogenesis. The ED-B isoform of fibronectin is of particular interest because of its restricted expression on normal adult tissues and its expression on tumor stroma and neoformed tumor vasculature. This has led to the concept that ED-B is associated with angiogenesis (18). However, the source of ED-B FN in tumors is unknown, although it has been suggested that the tumor cells themselves could produce the molecule (19). The association with angiogenesis might point to the endothelial cells. Transformed fibroblasts have been shown to make ED-B FN (16), but no other ED-B-expressing cell line has been identified.

To come to a fuller understanding of the role of ED-B FN in neoplastic disease, we investigated the location and source of ED-B FN. We used a variety of tumor- and endothelial-derived cell lines, as well as human breast and colorectal carcinoma tissue samples. Finally, we used a human tumor xenograft model to further define possible sources of ED-B FN in tumors.

MATERIALS AND METHODS

Cell Lines and Tissues. Human colon adenocarcinoma epithelial cells (HT 29; Ref. 20), oral epidermoid carcinoma cells (KB; Ref. 21), and breast carcinoma cells (MCF 7; Ref. 22) were grown in RPMI 1640 (Life Technologies, Paisley, United Kingdom) supplemented with 10% heat inactivated FCS (Globefarm, Surrey, United Kingdom), 100 IU/ml penicillin and 100 μg/ml streptomycin (Life Technologies), 2 mm L-glutamine (Life Technologies), and 1 mm sodium pyruvate (Life Technologies). HUVECs (23) were grown in flask precoated with 2% bovine gelatin (Sigma, Dorset, United Kingdom) in M199 (Life Technologies) supplemented with 20% heat-inactivated FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin, 2 mm L-glutamine, 12 units/ml preservative-free heparin (CP Pharmaceuticals, Wrexham, United Kingdom), and 100 μg/ml endothelial cell growth supplement (Sigma). Spontaneously transformed HUVECs (ECV 304; Ref. 24) and HUVECs fused with a human lung carcinoma cell line (EA hy 926; Ref. 25) were grown in the same complete medium as the tumor cell lines but without sodium pyruvate. All of the cells were incubated at 37°C in 4.5% CO2. The cells were split 1:4 weekly using 2 ml of sterile trypsin/EDTA dissociation solution (Sigma). Ten colorectal and 20 breast carcinomas of mixed histotypes were obtained from patients undergoing surgery for removal of these tumors. Tissue samples were snap frozen in liquid nitrogen shortly after removal and stored at −80°C. To
generate a KB tumor xenograft, KB cells were cultured, and 100 μl of the culture, containing 10^6 cells/ml, were injected s.c. into the flank region of "nude" (nude) 8- to 12-week-old female mice. Tumors were allowed to grow for 4–5 weeks. They were then excised and snap frozen in liquid nitrogen.

**Immunohistochemistry.** Indirect immunoperoxidase staining of either cryostat tissue sections (6 μm) or cytofissins was performed. The cytofissins were prepared using a Cytospin 2 (Shandon, Cheshire, United Kingdom) at 1400 rpm for 4 min. The slides were dried at room temperature. Tissues were fixed in acetone for 10 min, whereas cytofissins were fixed in acetone:methanol (1:1, v/v) for 1 min. Tissue sections and cells were incubated with the primary antibody for 1 h at room temperature. The optimal concentrations of all antibodies used were assessed by titration. BC-1 (kindly provided by Dr. L. Zardi; Ref. 26) is a mouse IgG1 monoclonal antibody, and was used to detect ED-B FN at a concentration of 24 μg/ml. The rabbit antihuman FN (DAKO, Glostrup, Denmark) antibody was also used at 24 μg/ml. After being rinsed in PBS, the slides were incubated with the secondary antibodies. Peroxidase-conjugated swine antirabbit immunoglobulins (DAKO) and rabbit antimouse immunoglobulins (DAKO) were used at concentrations of 26 and 13 μg/ml, respectively. The slides were counter-stained in hematoxylin (Sigma) for 30 s, rinsed in slowly running tap water for 3–4 min, and mounted in water-based mounting agent (Kaiser's).

**RT-PCR.** mRNA extraction and cDNA synthesis were as follows. mRNA was extracted from cell pellets of 1 × 10^6 to 1 × 10^7 cells, using a Quick Prep Micro mRNA Purification kit (Pharmacia P-L Biochemicals Inc., Herts, United Kingdom), and stored at −80°C. mRNA was extracted from tissues, following a similar procedure. Tissue samples (up to 0.1 g) were homogenized with 0.4 ml of extraction buffer on dry ice, using a diethyl pyrocarbonate-treated pestle and mortar of 2 cm<sup>3</sup> capacity. The RNA was heated for 10 min at 65°C and then chilled on ice. The First-Strand cDNA Synthesis kit (Pharmacia P-L Biochemicals) was used to prepare cDNA. The mixture was incubated for 90 min at 37°C.

**Primers.** A pair of primers (ED-B 5′/ED-B 3′) were designed and bought from Life Technologies to amplify the ED-B domain. β-actin was used as positive control, and appropriate primers were bought from Cruachem (Glasgow, United Kingdom).

The sequences of the primers were as follows:

ED-B 5′: CCATCATCCAGGATGCCCACACT
ED-B 3′: AGGAGAACACCGCTTGTTGGTGT
β actin 5′: GTGGGGCCGCACGGCACACA
β actin 3′: CTCTTAAATGTCAAGCAAGATTCC

ED-B 5′ was designed to contain the last 11 nucleotides of repeat 7 and the first 14 nucleotides of the ED-B exon, whereas ED-B 3′ contained the last 13 nucleotides of ED-B and the first 12 of repeat 8.

**Reactions.** Fifteen μl of cDNA were diluted to a final volume of 50 μl, which contained 20 pmol of each primer set. “Hot-start” PCR was performed, and 2.5 units of Taq polymerase (Life Technologies) were added in the PCR mixture as soon as tube temperature reached 94°C. Amplification was performed for 35 cycles (94°C for 1 min, 65°C for 1 min, 72°C for 2 min) using an Omnisense thermal cycler (Hybaid Limited, Middlesex, United Kingdom). PCR products were run on 1.5% agarose (Life Technologies) gel together with either a 1-kb or 1-kb plus molecular weight marker (Life Technologies).

For sequencing, the amplified DNA of several PCRs was precipitated using formamide buffer and ethanol, and was loaded onto a 373 DNA sequencer STRETCH (Applied Biosystems, Cheshire, United Kingdom).

**RESULTS**

**Distribution of ED-B FN Isoform in Malignant Tissue and Tumor Cell Lines.** To study the distribution of ED-B FN, immunohistochemical analysis was performed on cytofissins of the breast carcinoma cell line (MCF 7) and the colon adenocarcinoma cell line (HT 29). The BC-1 monoclonal antibody, which recognizes human ED-B-containing FN molecules, was used. ED-B-containing FN was detected in MCF 7 cytofissins but not in HT 29 (Fig. 1). FACS analysis was performed for these cell lines, but no ED-B was detected on cell surfaces (data not shown). Immunohistochemical analysis was also performed for tissue samples of both colorectal and breast tumors of mixed histotypes. Six of the 10 colorectal tumors (mixed histotypes) were positive for ED-B FN. The normal colon did not show any staining. Fig. 2 shows a representative colorectal adenocarcinoma. ED-B-containing FN was located in the epithelial cells of the tubular glands lining the intestinal mucosa of the colorectal adenocarcinoma (Fig. 2A). In contrast, FN lacking the ED-B domain was expressed mostly in the basement membrane and in the stroma surrounding the mucosa but was absent from the epithelial cells of the glands (Fig. 2B). No staining was seen in the negative control, with the exception of neutrophils containing endogenous peroxidase (Fig. 2C).

Twenty breast tumors of mixed histotypes and grades were tested by immunohistochemistry. ED-B FN was expressed in 19 of 20, representing 95% of the samples. The majority of the tissue sections again showed a predominately intracellular pattern of staining. In addition, in some cases, expression was also seen in the tumor stroma. There was no obvious association between staining intensity/pattern and tumor histotype in this small sample size. We did not observe any staining of blood vessels in any sample. The distribution of ED-B FN in a poorly differentiated infiltrating ductal grade 3 breast carcinoma is shown in Fig. 3A.

**RT-PCR Detection of ED-B FN mRNA Isoform in Human Colorectal and Breast Tumor Cell Lines and Tissues.** The presence of ED-B FN mRNA was studied in human tumor tissues and cell lines by means of RT-PCR using oligonucleotide primers. These were designed to contain sequences from both the ED-B FN exon and the
appropriate adjacent exons, thus assuring that genomic DNA would not be amplified. This was confirmed in experiments in which reverse transcriptase was omitted (data not shown). Furthermore, we performed experiments with primers based on exons 7 and 8 with similar results (data not shown), although these were complicated by amplification of the ED-B lacking FN sequences. The ~300 bp ED-B sequence was detected in both HT 29 and MCF 7 cells, as shown in Fig. 4A, Lanes 2 and 3, respectively. The presence of mRNA encoding ED-B FN in HT 29 cells coupled with the absence of ED-B staining in these cells indicates either that the message was not translated or that the product was secreted too rapidly to allow detection in the cytoplasm. The ED-B FN mRNA isoform was also detected in both human breast and colorectal adenocarcinoma biopsies (Fig. 4B, Lanes 2 and 3, respectively), but no ED-B FN mRNA was found in normal colon or lymph nodes (data not shown). We were unable to obtain any samples of normal breast. The identity of the amplification products was confirmed by sequencing of the PCR products. The resulting sequence matches the published sequence for ED-B FN (9).

Expression of ED-B FN mRNA Isoform in Normal and Transformed Endothelial Cell Lines. The RT-PCR analysis of the ED-B FN mRNA isoform was extended to primary and transformed endothelial cell lines. HUVECs from primary cell culture (Fig. 4C, Lane 2), EA hy 926, a hybridoma made from HUVECs and a human lung carcinoma cell line (Fig. 4C, Lane 3), and ECV 304, a spontaneously transformed HUVEC cell line (Fig. 4C, Lane 4), were shown to express the ED-B FN mRNA isoform by RT-PCR.

Study of ED-B FN in Human Tumor Xenografts. To study the production of ED-B FN in vivo, we used a tumor xenograft of the KB cell line (human oral epidermoid carcinoma). Immunohistochemistry

Fig. 2. Immunohistochemical analysis of colorectal adenocarcinoma. Indirect immunoperoxidase staining was performed on cryostat sections of colorectal adenocarcinoma to study the expression of ED-B FN by use of the monoclonal antibody BC-1. A, tubular glands stained with BC-1 in colorectal adenocarcinoma (×200). Epithelial cells are strongly positive for ED-B FN, whereas the connective tissue does not react with BC-1. B, colorectal adenocarcinoma stained with an antibody against FN lacking ED-B domain (×200). FN lacking ED-B domain is localized in the basement membrane and in the stroma surrounding the mucosa but is absent from the epithelial cells of the gland. C, negative control (primary antibody omitted), which shows that the area of the tubular gland in colorectal adenocarcinoma is not labeled (×200). Some endogenous peroxidase is seen in neutrophils.

Fig. 3. Immunohistochemical analysis of ductal grade 3 breast carcinoma. Indirect immunohistochemistry was used to detect the presence of ED-B FN in cryostat sections (6 μm thickness) of ductal breast carcinoma. A, ductal grade 3 breast carcinoma stained with BC-1 (×200). ED-B FN is localized mainly in and around the tumor cells. B, negative control (primary antibody omitted) of ductal grade 3 breast carcinoma (×200).
of KB cytospins and tumor xenograft tissue sections as well as RT-PCR in both cells and tissue were performed. Fig. 5A shows KB cytospins stained with BC-1, with ED-B localized in the cytoplasm and with some faint staining close to the plasma membrane. FACS analysis showed no surface expression of ED-B FN (not shown). In the KB xenograft, there was intense staining around tumor cells and...
in the tumor stroma (Fig. 5B), BC-1 antibody detects human but not murine ED-B-containing FN (27). Therefore, detection of ED-B FN in the tumor xenograft indicates that it was produced by tumor cells. The mRNA encoding ED-B-containing FN was present in both KB cells and the xenograft as detected by RT-PCR (Fig. 5C, Lanes 2 and 3).

**DISCUSSION**

The aim of this study was to investigate the source and location of ED-B FN expressed in tumors because this has clear implications for both the development of immunotherapeutic approaches targeting ED-B FN and the development of our understanding of the role of this molecule in tumorigenesis.

The source of ED-B FN in tumors is unknown, although previous workers have speculated that it might be produced by the tumor cells themselves (19, 28). Transformed fibroblasts have been shown to express ED-B FN (16). We analyzed tumor cell lines derived from colorectal (HT 29), breast (MCF 7), and head and neck (KB) carcinomas, and human primary endothelial cells (HUVEC) and endothelial cell lines (EA by 926 and ECV 304). All of these cells expressed ED-B FN (as determined by RT-PCR), indicating that both tumor and endothelial cells could be the source of ED-B FN in tumors.

To investigate the contribution of neoplastic cells to ED-B FN in tumors, we stained human KB tumor xenografts grown in mice with the BC-1 antibody (which does not recognize murine ED-B FN). Strong staining both around the tumor cells and in the stroma indicates that the tumor cells can act as the source of ED-B FN in tumors, although it does not rule out a possible contribution by endothelial cells.

We have further investigated the cellular location of ED-B FN expression. Staining of cytopsins of the cell lines indicated that in some of them ED-B FN could be detected in the cytoplasm. However, no staining was seen on the plasma membrane (as determined by FACS analysis). This indicates that ED-B FN is not expressed on the cell surface but is secreted from the cell. The failure to detect ED-B FN in the cytoplasm of some of the cell lines despite the clear presence of mRNA was presumably due to the rapid secretion of the molecule.

This study was complemented by immunohistochemical analysis of both breast and colorectal carcinomas. The BC-1 antibody stained 95% of the samples with breast carcinoma, which represented a variety of different histotypes. We observed submembranous and membranous staining of tumor cells. Kaczmarek *et al.* (17), in a larger study of invasive ductal cell carcinoma, also observed staining of the blood vessels, as has also been seen for glioblastoma multiforme, high-grade astrocytoma, and benign tumors such as ependymoma and breast intra ductal papilloma (18). Our failure to observe similar staining of the vasculature may be a result of the many different tumor histotypes and small sample size we analyzed. In colorectal carcinoma samples, we saw staining associated with both the tumor cells and the tumor stroma in 60% of samples, similar to that reported previously (19). We observed no staining of the vasculature; expression of ED-B FN by the vessels was not commented on by the authors of the previous study (19).

These data, when taken together, suggest that tumor cells can act as a major source of ED-B FN in tumors. The cells do not express ED-B FN on their surface, but secrete it. Once secreted, it can contribute to the ED-B FN seen in the tumor stroma. In tumor samples, ED-B FN can be seen closely associated with the tumor cells. This may reflect secreted ED-B FN that forms part of the stroma immediately surrounding the tumor cells. This has implications for the use of ED-B FN in tumor therapy or in vivo diagnosis. Early reports showed ED-B FN expression in the lumen of blood vessels (13, 18), which would therefore be highly accessible to antibody-mediated therapy or imaging. Our data and those of others (17) indicate that this is not the case for all types of tumors. This reduces the attractiveness of using IgG antibodies because it will be necessary for these large molecules to penetrate the vasculature. To this end, we have constructed a single-chain Fv version of the antibody (29), which should show better penetration of solid tissue for tumor imaging and therapy.

Clearly, the lack of surface expression of ED-B FN influences the possible forms of therapy. Thus, immunotoxin therapy, which requires direct binding of the therapeutic agent to the tumor cell, would not be feasible. However, either radioimmunotherapy or antibody-directed enzyme prodrug therapy might be effective with antibodies targeted to stroma rather than the tumor cells.

The lack of staining of endothelial cells in blood vessels is perhaps surprising in light of the detection of ED-B mRNA in HUVEC cells. There are several possible explanations for this. First, HUVEC cells growing in vitro will not represent the tumor-associated endothelium where division will be less; unfortunately, these are not good in vitro models for this situation. Second, the relative rates of production of ED-B FN are not known. Finally, ED-B FN is secreted and may then be laid down in extracellular matrix some distance from the cells producing the molecule.

To date, the role of the ED-B repeat in the fibronectin molecule has been unknown. However, there is evidence to suggest that it might play a role in, or affect, cell adhesion and spreading (30, 31). Both cell adhesion and cell spreading are important phenomena in embryogenesis, wound healing, angiogenesis, tumor establishment, and metastasis. No ligand has yet been identified for the ED-B domain. It has been suggested that insertion of ED-B into fibronectin causes conformational changes in the molecule (26), improving access to the integrin binding sites in the 6th and 10th type III repeats. The finding that both tumor and endothelial cells express ED-B FN is clearly important for the investigations of the role of this molecule in tumor growth, establishment of metastasis, and angiogenesis.

**ACKNOWLEDGMENTS**

We thank Dr. Luciano Zardi (Laboratory of Cell Biology, Genoa, Italy) for the generous gift of BC-1 (the monoclonal against ED-B FN).

**REFERENCES**


Source of Oncofetal ED-B-containing Fibronectin: Implications of Production by Both Tumor and Endothelial Cells

Marta Midulla, Rakesh Verma, Massimo Pignatelli, et al.