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FAST TRACK

Monoclonal Antibody to Fibulin-1 Generated by Genetic Immunization

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Abstract Fibulin-1 (Fbln-1) is an extracellular matrix (ECM) and plasma glycoprotein. Considering the growing evidence indicating that Fbln-1 plays a role in cancer we sought to develop monospecific antibodies to better facilitate further studies of the function of Fbln-1 in breast cancer. Using a plasmid expression vector encoding full-length human Fbln-1D as an immunogen and CpG oligodeoxyribonucleotides as adjuvant a monoclonal antibody (MAb) against Fbln-1 was produced. This MAb, designated MEM-2 was of IgM isotype and reacted with bacterially expressed Fbln-1. Furthermore, MEM-2 reacted with Fbln-1 expressed in the ECM released by cultured human breast carcinoma SKBR-3 cells in ELISA, and also with Fbln-1 present in SKBR-3 cell extract in immunoprecipitation and Western blotting. MEM-2 also reacted with Fbln-1 in human breast carcinoma specimens. These findings illustrate the utility of genetic immunization as a means of generating monoclonal antibodies to tumor-related ECM proteins. MEM-2 represents a useful new tool for the study of Fbln-1 in breast cancer. J. Cell. Biochem. 89: 647–652, 2003.

Key words: Fibulin-1; monoclonal antibodies; genetic immunization; CpG oligodeoxyribonucleotides

Fibulin-1 (Fbln-1) is the prototypic member of a family of extracellular matrix (ECM) and blood glycoproteins [Argraves et al., 1990].

Alternative splicing of Fbln-1 precursor RNA results in the expression of four transcripts which encode overlapping polypeptides, designated A–D, differing only in their carboxy terminal domain.

The Fbln-1 glycoprotein can self-associate and bind to ECM proteins including fibronectin, laminin, and nidogen, and to the coagulation protein fibrinogen [Hayashido et al., 1998]. Immunohistological analysis of human tissues revealed that Fbln-1 is a widely expressed protein often associated with basement membranes and matrix fibers particularly those containing elastin [Roark et al., 1995].

There is emerging evidence that Fbln-1 has a role in cancer. Two recent articles report evidence that Fbln-1 expression is elevated in breast tumors [Pupa et al., 2002b; Greene et al., 2003].

It has been shown that Fbln-1 is secreted by several estrogen-receptor-positive ovarian cancer cell lines and that its production is stimulated by estrogens, thus facilitating ovarian tumor cell invasion [Clinton et al., 1996]. By reverse transcription-polymerase chain reaction (RT-PCR) analysis, we found high levels of Fbln-1 transcripts in breast carcinomas from surgical specimens and cell lines and in ovarian and lung carcinoma cell lines but not in melanoma [Forti et al., 2002], consistent with the previous suggestion that this molecule is overexpressed only in some cancer histotypes [Clinton et al., 1996]. In our serological analysis of cDNA expression library (SEREX), we found a statistically significant breast cancer-related humoral immunity to Fbln-1 and we suggested that this specific antibody immunity could be exploited as tool for early detection of breast cancers [Forti et al., 2002; Pupa et al., 2002a]. Successively, we also detected upmodulation of

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Fbln-1 transcripts after doxorubicin and thermal shock treatments at 42°C of breast tumor cell lines [Pupa et al., 2002b]. Since additional experiments are required to elucidate the role of Fbln-1 molecule in the anti-tumor immunogenicity, growth and progression and response to anti-cancer therapy, reagents suitable to study these cellular mechanisms and to discriminate the involvement of the different protein isoforms are mandatory. In the present work, we describe, for the first time, the production and characterization of a mouse monoclonal antibody directed against Fbln-1 using a pcDNA-3 plasmid vector encoding the human Fbln-1D and CpG olygodeoxynucleotides (ODNs) as immunizing material [Krieg, 2002].

MATERIALS AND METHODS

Tissues and Cell Culture Conditions

Primary breast tumor specimens were obtained from breast cancer patients treated at the Istituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy. Information on tumor histology was obtained from histopathological reports.

The human breast carcinoma cell line SKBR-3 was obtained from ATCC (Rockville, MD). Cells were grown in RPMI 1640 (Sigma, St. Louis) containing 10% (v/v) fetal calf serum (Sigma) and L-glutamine in a humidified chamber (95% air and 5% CO_2) at 37°C.

Production of Anti-Fbln-1 MAb

The pcDNA3- Fbln-1D plasmid vector has been described and propagated into *E. coli* XL1-Blue cells. Large-scale preparation of the plasmid was carried out according to Maxiprep DNA purification system (Quiagen) and insert size was checked by *Xho*I-*Xba*I restriction enzyme digestion.

Two Balb/c mice were inoculated intradermally in the ear pinna using 32-gauge needles four times at 3-weeks interval with a total of 100 μg of pcDNA3− Fbln-1D naked DNA plus 5 μg of CpG-ODNs (M-Medical-GENENCO, Firenze, Italy), prepared as described [Forg et al., 1998], diluted in 50 μl of sterile phosphate-buffered saline (PBS) for injection. Before each manipulation, mice were anesthetized with 0.2 ml per 20 g body weight of mg/ml ketamine and 0.05% xylazine. Mice were bleeded the second week after the last DNA injection and the mouse whose serum resulted to be more reactive by ELISA on deposited ECM preparation [Giannelli et al., 1999] was challenged with immunizing material 3 days before sacrifice. The splenocytes were fused with the NSO mouse myeloma line and seeded in 96-well plates. The hybridomas were grown in HAT medium and their supernatants were recovered for selection by ELISA. The hybridomas were maintained in pristane-pretreated Balb/c mice.

Selection of Anti-Fbln-1 MAb by ELISA

Undiluted supernatants from hybridomas and murine polyclonal immune serum diluted 1:100, tested as internal positive control, were incubated for 1 h at 37°C in 96-well plates precoated with deposited ECM derived from SKBR-3 tumor cell line and prepared as described [Giannelli et al., 1999]. After several washings with PBS, anti-mouse Ig horseradish peroxidase (HPR)-conjugated antibody was incubated for 1 h at 37°C. After additional washing, the immune complexes were detected by adding 3,3′,5′,5 tetramethylbenzidine (TMB) (Sigma) as substrate for 30 min at room temperature in the dark. The enzymatic reaction was arrested by adding 1 M H_2SO_4. Plates were read at 450 nm using an automated ELISA reader.

Immunoprecipitation and Western Blotting

SKBR-3 was lysed for 45 min on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, and 0.5% deoxycolic acid) containing protease inhibitors, 2 mM phenylmethylsulfonylfluoride (PMSF) and 10 μg/ml aprotinin. Lysate was clarified for 15 min at 15,000*g*. Protein concentration was determined by the BCA protein assay (Pierce Biochemical Co., St. Louis, MO). Cell lysate was precleared for 30 min with GammaBind Plus Sepharose (Pharmacia Biotech, Uppsala, Sweden), equilibrated in lysis buffer, by incubation on a rocker for 30 min at 4°C. Sepharoses (20 μl), equilibrated in lysis buffer, were incubated with MEM-2 MAb diluted 1:100, on a rocker for 2 h at 4°C. After three washings with lysis buffer, sepharose conjugated with MEM-2 was incubated with precleared lysate (1 mg protein/sample) on a rocker for 3 h at 4°C. After immunoprecipitation, immunecomplexes were washed three times with lysis buffer, eluted and denaturated by heating for 5 min at 95°C in reducing Laemmli

sample buffer and resolved in a 10% polyacrylamide gel. Separated proteins were electrophoretically transferred to nitrocellulose membrane (Hybond C, Amersham, Little Chalfont, UK) and incubated at room temperature for 1 h with MAb MEM-2 (1:200) followed by incubation at room temperature for 1 h with anti-mouse Ig biotinylated species-specific whole antibody (1:400) and streptavidin-horseradish peroxidase conjugate (1:400) (Amersham) and visualized using the ECL detection system (Amersham) according to the supplier's instructions.

Immunohistochemistry

Immunoperoxidase assay was carried out by a sensitive peroxidase-streptavidin method on formalin-fixed, paraffin-embedded sections of human breast carcinomas. Briefly, sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by 0.3% hydrogen peroxide in methanol for 30 min. After washes in Tris-buffered saline (TBS) and treatment with normal goat serum 1:50 for 30 min at room temperature, immunostaining was performed by incubation of 1:100 diluted MEM-2 ascitic fluid for 1 h followed by biotinylated anti-mouse IgG and HRP-conjugated streptavidin (Dako, Glostrup, Danmark). Peroxidase activity was detected by diaminobenzidine. Staining without MEM-2 was performed as negative control.

Expression of Fbln-1D in Bacteria

Recombinant Fbln-1 was cloned and produced according to the manufacturer's instructions provided in Affinity LIC Cloning and Protein Purification Kit (Stratagene, La Jolla, CA 92037). The system allows fusion of the calmodulin binding peptide (CBP) purification tag followed by the flag epitope to the N-terminus of the protein coding sequence of interest.

Briefly, the entire open reading frame of Fbln-1 (2,111 bp) was obtained by PCR amplification, using as template full-length cDNA cloned in pcDNA3.

PCR was performed using the following set of primers: (+) 5′-GACGACGACAAGATGGAGCGCGCCGCGCCGTCG-3′ and (−) 5′-GGAACAAGACCCGTTGAGAACCAGTACTCAGAGAC-3′ designed on 5′ and 3′ ends of the gene tailed with ss overhangs complementary to the vector pCAL-n-Flag-specific sequence to allow cloning.

After initial denaturation at 95°C for 5 min, 20 cycles at 95°C for 1 min, 62°C for 1 min and 72°C for 2 min were carried out followed by a final extention at 72°C.

Ligation independent cloning (LIC) was performed with 100 fmol of purified insert and 20 ng of the provided restriction-digested pCAL-n-Flag vector. The fusion construct was first transformed into *E. coli* XL10 Gold supercompetent cells to allow selection of recombinant clones. Positive clones were subcloned and identity was confirmed by restriction analysis and sequencing.

E. coli BL21 (DE3) expression strain was successively transformed with the fusion construct to allow expression of *CBP + Fbln-1* gene under the control of IPTG-inducible promoter. The pTC12 vector encoding for CBP-β-galactosidase protein was included as a positive control for induction of CBP fusion protein. Induction was performed in the presence of 1 mM IPTG for 5 h at room temperature in 1 ml-aliquots of LB on an analytical scale. Non-induced samples were included as negative control.

Non-induced and induced cell cultures were resuspended in 1× SDS sample buffer, boiled at 95°C for 5 min, and analyzed by SDS–PAGE under reducing conditions.

RESULTS

Production and Selection of MEM-2 MAb

Thirty-eight hybrid colonies were obtained from the fusion of NSO myeloma cells with spleen cells from a Balb/c mouse immunized with the pcDNA3-Fbln-1D variant plasmid vector plus CpG-ODNs as adjuvant.

An ELISA screen of the hybridomas was performed using microtiter wells coated with SKBR-3 tumor cell-deposited ECM (Fig. 1). Based on this screen, three clones were selected. The most reactive one of these, designated MEM-2 (hybridomas conditioned supernatant no. 5), was further characterized. Immunofluorescence analysis indicated that MEM-2 MAb was of IgM isotype (data not shown).

Western Blotting and Immunoprecipitation With MEM-2 on SKBR-3 Protein Extracts

An immunoblotting assay performed on soluble extract of SKBR-3 cells with the MEM-2 MAb showed that the antibody recognizes a band migrating approximately at 73 kDa corresponding to isoform D and a weakly staining

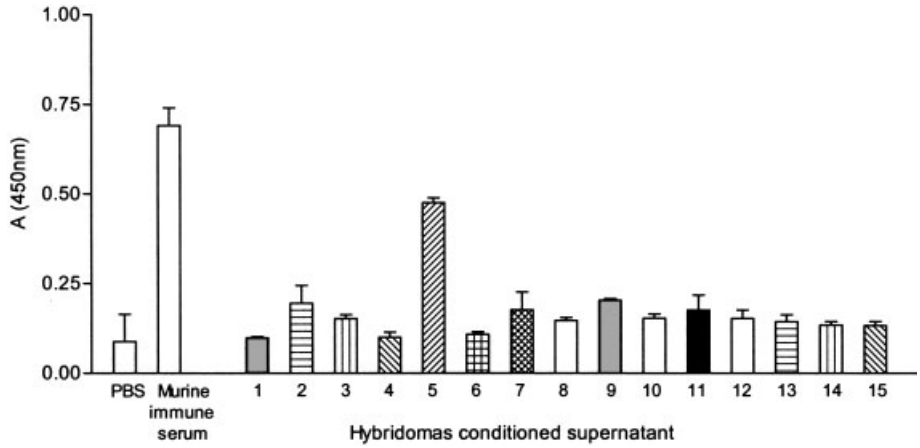

Fig. 1. ELISA screening of hybridoma supernatants. Undiluted culture supernatants from 15 hybridomas including MEM-2 (clone 5) were incubated with microtiter wells coated with SKBR-3 cell-deposited ECM. Each value represents the mean of triplicate readings of two independent experiments.

band at 70 kDa corresponding to isoform C. Other bands of lower Mr were evident that perhaps corresponded to Fbln-1-derived degradation products. Potential Fbln-1 dimers were also evident in the immunoblot (Fig. 2A, lane 1). Immunoprecipitation analysis of the SKBR-3 detected a band of approximatively 73 kDa (Fig. 2B, lane 1).

MEM-2 Immunohistochemistry Reactivity in Tumor Breast Cancer

MEM-2 antigen was detected by immunoperoxidase, reaction in both formalin- and Bouin-fixed paraffin-embedded sections from breast cancer specimens (Fig. 3) and in acetone-fixed breast carcinoma cell lines (data not shown). The antibody detected Fbln-1 expression in 8/21 breast cancer samples with prevalent staining apparent in the cytoplasm of the tumor cells.

MEM-2 Reacts With Fbln-1D Expressed in Bacteria

The CBP-Fbln-1 fusion protein was expressed in bacteria cells. As shown in Figure 4, MEM-2 reacted with ~80 kDa fusion protein expressed in crude extracts of the transformed bacteria grown in the presence of 1 mM IPTG. These

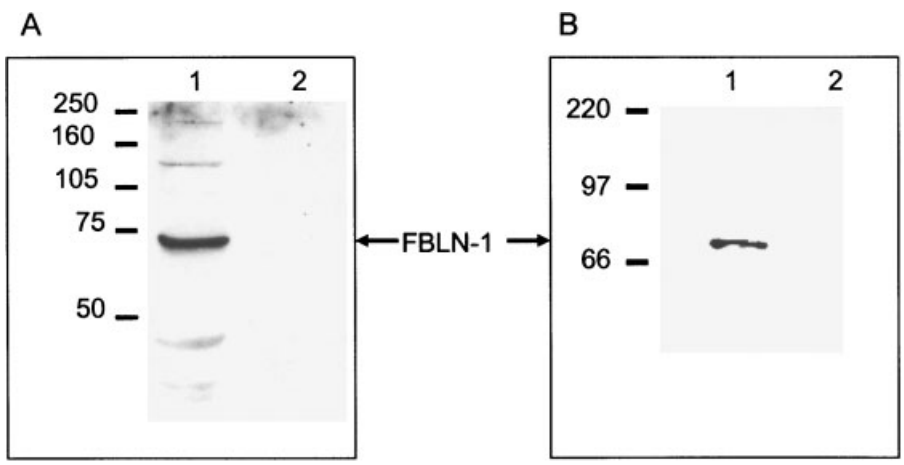

Fig. 2. Characterization of the specificity of MEM-2 MAb. **A**: Western blot analysis of SKBR-3 cell extract performed using MEM-2 MAb (**lane 1**) and pre-immune mouse serum at 1:100 dilution, as negative control (**lane 2**). **B**: SDS–PAGE on 10% gel of SKBR-3 cell extract immunoprecipitate with MEM-2 MAb (**lane 1**) and preimmune mouse serum (**lane 2**), as negative control. Immunocomplexes were electrophoretically blotted and incubated with MEM-2 MAb.

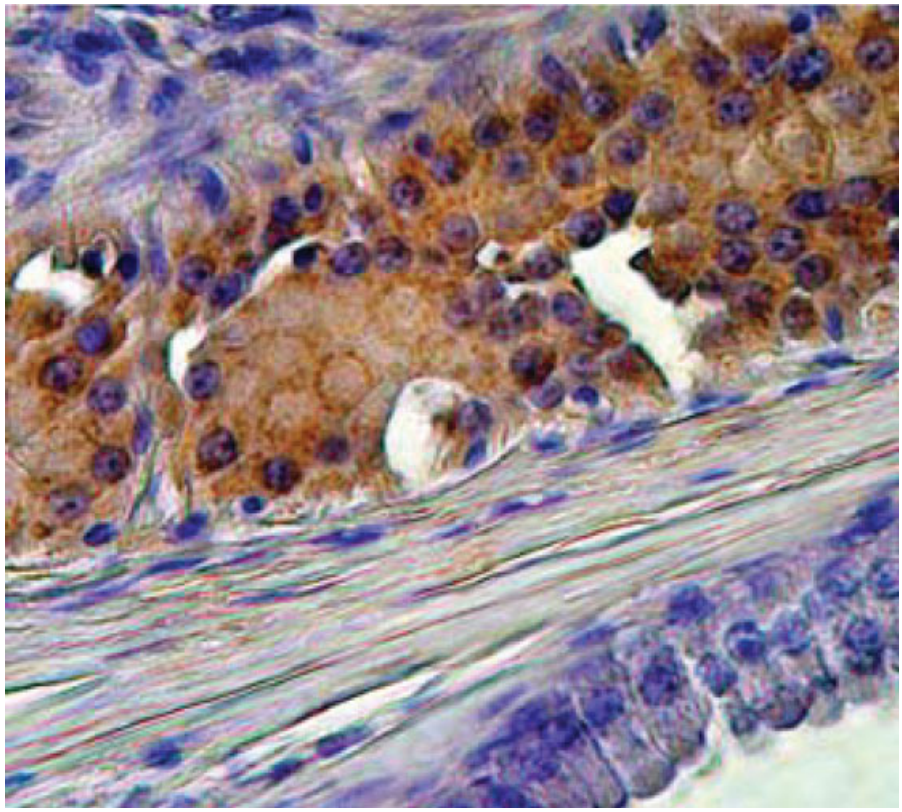

Fig. 3. Immunohistochemical analysis of formalin-fixed breast carcinoma tissues using MEM-2 MAb. Bar corresponds to 50 μm.

findings further support the conclusion that MEM-2 is an Fbln-1 specific MAb.

DISCUSSION

Using a plasmid construct designed to express human Fbln-1 as immunogen and CpG as adjuvant, a murine MAb specific for the fbln-1 was isolated. The MAb, designated MEM-2, is able to recognize both denaturated and native Fbln-1 protein. The expression construct used as immunogen encoded the Fbln-1D isoform which is differentially expressed in different tissues and tumors and has been shown to suppress cell motility and tumor growth [Qing et al., 1997; Twal et al., 2001; Du et al., 2002; Forti et al., 2002]. MEM-2 seems to recognize both the D and C isoforms. Additional experimentation

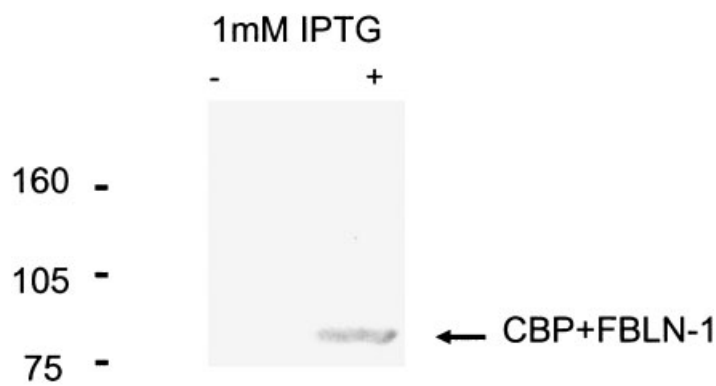

Fig. 4. Immunoblot analysis of bacterially expressed Fbln-1 using MEM-2 MAb. Extracts were made from cells cultured in the presence or absence of IPTG (1 mM).

is required to map the epitope, to confirm isoform specificity and to determine whether the antibody has any function blocking activity.

DNA vaccination protocol has great promise for the production of monoclonal antibodies. Muscle and skin have been described as optimal sites for DNA immunization. The dermis of the ear pinna, the immunization site used in this study, has been found to be a privileged site for induction of strong antibody and cellular anti-tumor immune responses as compared to the dermis of abdominal skin or skeletal muscle [Forg et al., 1998; Schirmacher et al., 2000]. The fact that the thin ear pinna has two layers of epidermis and dermis in effect doubles the amount of specialized antigen presenting cells including keratinocytes, macrophages and Langherans cells that can be involved in initiation and augmentation of immune responses [Gurunathan et al., 2000].

In conclusion, by using an immunization protocol that does not require a purified protein antigen, a MAb directed against a tumor-associated molecule Fbln-1 was generated. This MAb will facilitate studies that should extend our understanding of the role of Fbln-1 in processes such as: anti-tumor B- and T-mediated cells immunity, tumor and ECM interactions and tumor onset and progression.

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