Characterization of a novel mouse monoclonal antibody, clone 1E8.33, highly specific for human procollagen 11A1, a tumor-associated stromal component

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Abstract. A novel IgG1, κ mouse monoclonal antibody (clone 1E8.33) to human procollagen 11A1 has been generated. This antibody is poorly mutated, essentially in germ line configuration; its complementarity determining regions (CDRs) are especially rich in tyrosine and serine residues. The epitope recognized is encompassed in the YNYGTMESYQTEAPR amino acid stretch within the variable region of human procollagen 11A1. Human procollagens 5A1 and 11A1 are very similar. However, this antibody does not cross-react with human procollagen 5A1. In human breast tumors, only the activated peritumoral myofibroblasts show a strong intracytoplasmic staining with this antibody. As procollagen 11A1 is overexpressed in the stroma of human tumors with desmoplastic reaction, this antibody represents a valuable tool for diagnostic purposes.

Introduction

The growth of some solid tumors, especially carcinomas, is accompanied by the development of a significant desmoplastic reaction (1-6). This reaction is mainly built up by activated stromal myofibroblasts which have been shown to overexpress and secrete a variety of collagens, in particular in pancreatic ductal adenocarcinoma (7-11). As extracellular matrix proteins, these collagens show a shield-like arrangement that, in one hand, contributes to cancer progression, and, in the other hand, leads to chemoresistance (12-14).

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Among the tumor-associated matrix collagens, fibrillar collagens are the most conspicuous. In normal tissues, collagen types I, II and III are the main major fibrillar collagens while types V and XI are less abundant minor fibrillar collagens (15).

Each collagen protomer is usually made of three different polypeptides, designed as α1, α2, and α3, and coded by specific gene sequences. These polypeptides are synthesized as procollagens which include the N- and C-propeptides flanking the prototypical collagen triple helix. Once secreted, the propeptides are excised by proteolytic cleavage and then the mature collagen molecules assemble extracellularly in fibrils.

Collagens V and XI are very similar and their procollagen polypeptides can intermingly associate in heterotypic protomers. A wealth of recent reports indicate that collagens V and/or XI, the genes encoding for COL5A1 and COL11A1 proteins, are overexpressed in the stroma of some human tumors (16-31). The main nucleotide and amino acid sequence differences between procollagens COL5A1 and COL11A1 fall in the so-called variable region within the N-propeptide. The development of immunological tools highly specific for each of those procollagens is of a great interest for diagnosis and potentially for therapeutics. We have previously described the generation of a rabbit polyclonal antiserum to the variable region of human procollagen 11A1 (21). We now report the generation of the novel mouse monoclonal antibody (mAb) the 1E8.33 clone, to the variable region of human procollagen 11A1, and its sequence characteristics, epitope mapping and immunohistochemical performance.

Materials and methods

Mouse immunization and hybridoma generation. The DNA sequence encoding the 133 amino acid stretch (E268 to E400) within the variable region of human procollagen COL11A1

(Fig. 1) was cloned and expressed as a GST fusion (proCOL11A1-T-GST) (22). The purified recombinant product was used to hyperimmunize BALB/c mice.

Using Sp2/0 myeloma cells as fusion partner, B-cell hybridomas were generated by standard methods; their antibody-containing supernatants were screened by ELISA against purified proCOL11A1-T-GST. Mouse antibody subisotyping was done by means of the IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Diagnostics GmbH, Mannheim, Germany).

Antigens and antibodies. Purified recombinant human procollagen COL5A1 (proCOL5A1) was kindly provided by Professor Florence Ruggiero, Lyon, France (32,33). Purified recombinant His6x-human proCOL11A1-T (lot 080429 5#7) fusion protein with no GST from a pET-46 EK/LIC (71335-3, Novagen) construction and purified ABK-Mab 1E8.33 (lot 100119 #1/1) were provided by Abyntek, Parque Tecnológico de Zamudio, Derio, Vizcaya, Spain. Rabbit polyclonal to collagen V (ab7046, lot 878902) was from Abcam.

V gene sequencing. The nucleotide sequencing of the VH and VL domains of the 1E8.33 mAb was performed as already described (34), using amplification primers specific for mouse IgG1 and κ chains.

Nucleotide sequences were checked by means of Chromas Lite 2.01 (Technelysiun Pty Ltd.) and Bioedit Sequence Alignment Editor 7.0.9 (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA), and analyzed through IMGT/V-QUEST (http://imgt.cines.fr) (35) and IgBLAST searches of GenBank databases (http://www.ncbi.nih.gov). The 5' ends of the sequences were rewritten, taking as template the closest germ line, omitting the nucleotide changes introduced by the primers used for amplification. VH and VL nucleotide sequences have been deposited in the EMBL Nucleotide Sequence Database with accession numbers HE608248 and HE608249, respectively.

Cell cultures. The HTB-82 (A204), HTB-85 (SAOS-2) and CRL-1690 (T98G) cell lines were obtained from the ATCC. They were cultured in DMEM supplemented with sodium pyruvate, L-glutamine, non-essential amino acids and 10% fetal bovine serum.

Passages and cell collections were done by trypsinization. For immunohistochemistry, cells were cultured in 4-well cultures slides (BD Falcon™, ref. 354114); for Q-RT-PCR, fresh cell pellets were kept at -80˚C.

Human tissue samples. All samples were obtained from the Banco de Tumores, Hospital Universitario Central de Asturias, after written informed consent of the patients and approval by the Ethics Committee of Clinical Research of the Principado de Asturias, Oviedo, Spain. Freshly removed tissue samples were maintained at -80˚C or fixed in formalin and embedded in paraffin.

Q-RT-PCR. Quantitative RT-PCR of COL5A1, COL11A1 and PUM1 mRNA was performed as already described (22,28). Briefly, total RNA was isolated from cell cultures and biopsies, kept at -80˚C, with RNeasy Mini kit (Qiagen). cDNA was synthetized using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time Q-PCR reactions were prepared using the TaqMan Gene Expression Master Mix kit and TaqMan Gene Expression Assays (Applied Biosystems). Data were normalized for each and every cell line and tumor sample relative to the values of the control normal samples. The gene expression ratio (SLR, signal log ratio) was determined by applying the ΔCt method, after PCR efficiency corrections.

Western blot assays. To study potential cross-reactivity, 10 μg/lane of purified recombinant human proCOL5A1 and His6x-human proCOL11A1-T were subjected to 8% polyacrylamide SDS-PAGE under reducing conditions and, subsequently, electrotransferred onto nitrocellulose membranes. After blocking overnight at 4˚C in PBS (10 mM phosphate-buffered saline)-3% BSA-0.1% sodium azide (SA), the membranes were then incubated with mAb 1E8.33 (3 μg/sample lane, in PBS-1% BSA-0.1% Tween-20), with gentle rocking for 2 h at room temperature. After several washing steps, blots were incubated with a 1:100,000 dilution of anti-mouse γ chain specific-HRPO-conjugated (Sigma A3673) in PBS-1% BSA-0.1% Tween-20, for 2 h as above, and finally developed with the Immobilon western chemiluminiscent HRP substrate (Millipore).

A204, SAOS-2 and T98G cells, collected from tissue culture flasks, were directly lysed in SDS-PAGE sample buffer and subjected to 8% polyacrylamide SDS-PAGE under reducing conditions. The rest of the assay proceeded as above. The membranes were incubated with either the 1E8.33 mAb, probed at 3 μg/lane; or with the rabbit polyclonal to collagen V, 1:2,000, in PBS-1% BSA-0.1% Tween-20.

Epitope mapping using a peptide scan. An N-acetylated peptide scan (peptide length: 15 amino acid residues; overlap: 12 amino acid residues), covering 155 amino acid residues from E255 to T409, encompassing the variable region of human proCOL11A1, was prepared by SPOT-synthesis on a cellulose membrane by JPT Peptide Technologies GmbH, Berlin, Germany.

Following the manufacturer's instructions, the 48 peptide membrane was first probed with the 1E8.33 mAb, and, after regeneration, with just the secondary anti-mouse γ chain-specific-HRPO conjugate (Sigma A3673) 1:50,000 in PBS-1% BSA.

Immunohistochemistry (IHC) (21). Cell culture slides were first fixed with ethanol, and tissue samples with formalin. Slides or paraffin-embedded tissue sections were stained with H&E for histological examination. For immunohistochemistry, the EnVision method (Dako) was applied. The 1E8.33 mAb was assayed at 1:700, and the rabbit polyclonal to collagen V at 1:500, both in EnVision™ FLEX Antibody Diluent (DM830, Code K8006, Dako) (Dako Diluent).

Western blot and IHC blocking tests with soluble peptides. Synthetic peptides, >/95% pure, were supplied by PolyPeptide Laboratories France SAS. For Western blot blocking tests, 300 μl of the 1E8.33 mAb, at 1:350 (~10 μg/ml) in Dako Diluent, were mixed with an equal volume of diluted synthetic peptide at 1,000 μg/ml in Dako Diluent. In the positive control samples, the addition of the competitor peptide was omitted; the negative control was the Dako Diluent. Then, the mixtures were preincubated for 2 h at 37˚C and overnight at 4˚C.

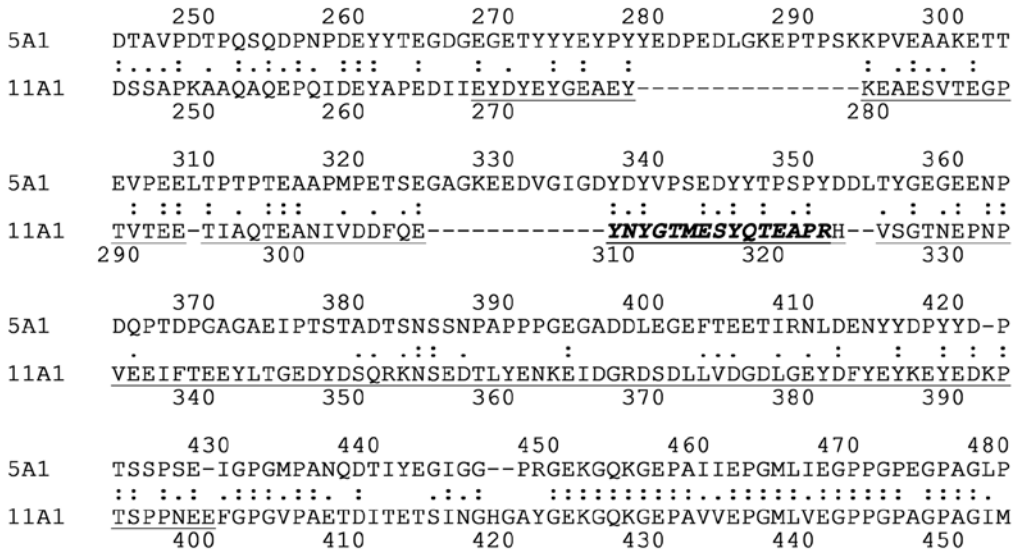

Figure 1. Comparison of the sequences within the variable region of human procollagen COL5A1 and COL11A1 using Lalign v2.0. The underlined stretch was chosen as the most distinct of COL11A1. The location of the YNYGTMESYQTEAPR stretch is highlighted in bold-faced italic.

Table I. Gene segment characterization and amino acid substitutions of VH and VL regions.

	Germ line genes		Amino acid changes					
1E8.33 mAb	V_H	J_H	FR1	CDR1	FR2	CDR2	FR3	CDR3
VH	IGHV1S34*01	IGHJ4*01	1 A17>S	0	1 H46>Q	1 S57>N	3 Q69>R K70>N E97>G	0
VL	IGKV4-50*01	IGKJ1*01	0	0	0	0	1 A100>D	0

For IHC blocking tests, 100 μl of the 1E8.33 mAb were incubated overnight as described above, with 100 μl of diluted synthetic peptide at 1,000 μg/ml in Dako Diluent. A volume of 200 μl of these mixtures were applied onto human breast tumor slides.

Results

Gene and CDRs characteristics. The 1E8.33 antibody is an IgG1, κ mAb. The gene and amino acid residue characteristics of this antibody are shown in Table I. In comparison with their closest germ lines, both mature VH and VL domains show rather limited amino acid changes in spite of having been rescued from a mouse which was extensively immunized with a proteic immunogen.

Table II shows the CDR characteristics of this mAb. These CDRs are especially rich in tyrosine (18% of the total CDR composition) and serine (13%) residues, which are highly abundant in natural antigen-binding sites (36,37).

Accordingly to available data, the mature VH of the 1E8.33 mAb has not been previously found in databases. The VH nucleotide sequence shares a 96.92% homology with that of an antibody which recognizes GM1 ganglioside-bound amyloid b-protein (no reference). The VL one is 99% homologous to that

Table II. The CDR characteristics.

	Amino acid residues in CDR1-CDR2-CDR3		
1E8.33 mAb	CDR1	CDR2	CDR3
VH 8-8-12*	G**YS**FTG**YY**	INC**Y**NGAT	AIWD**Y**EFHVMD**Y**
VL 5-3-9	**SS**VN**Y**	**Y**T**S**	QQFT**SS**PWT

*Number of amino acid residues in CDR1-CDR2-CDR3. Tyrosine (Y) and serine (S) residues are highlighted in bold.

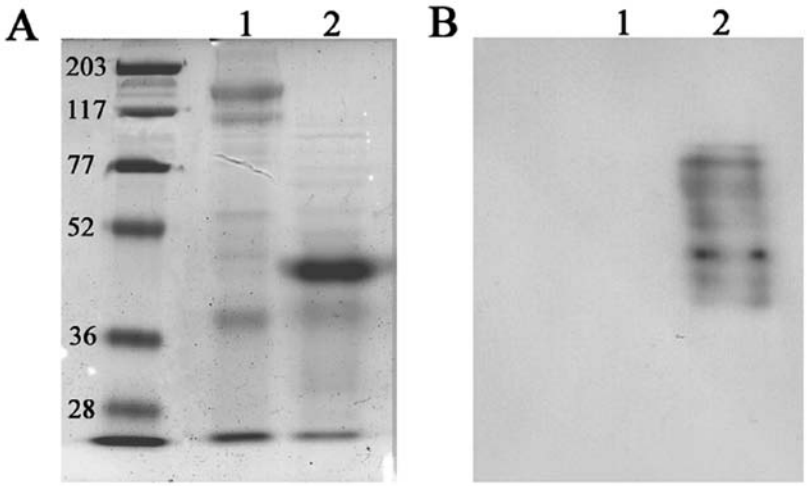

Figure 2. Analysis of cross-reactivity. (A) Coomassie blue-stained SDS/polyacrylamide (8%) gel; (B) Western blotting with the 1E8.33 mAb. Molecular weight markers (kDa) in the left lane. Lane 1, purified recombinant proCOL5A1 (10 μg/lane). Lane 2, purified recombinant His6x-proCOL11A1-T (10 μg/lane).

of an antibody raised against the LDL receptor. Thus, as a whole, the 1E8.33 mAb represents a novel antibody not reported so far.

Study of cross-reactivity of the 1E8.33 mAb on human procollagen 5A1 by Western blotting. As shown on Fig. 2, under the above mentioned experimental conditions, the 1E8.33 mAb only recognizes His6x-proCOL11A1-T, not proCOL5A1. Therefore, according to this observation, the 1E8.33 mAb does not react with human procollagen 5A1.

Expression of human procollagen 11A1 in human tumor cell lines. The human rhabdomyosarcoma A204 cells have been reported to mainly synthesize COL11A1 as the major collagen mRNA (38); we chose this cell line as a positive control for the expression of human procollagen 11A1. By contrast, the human osteosarcoma SAOS-2 mainly synthesises collagens of type I and V, and the COL11A1 chain to a much lesser extent (39,40). The T98G glioblastoma multiforme cells were also probed because human glioma tissues and cultured glioma cells have been shown to overexpress the COL11A1 gene (41,42).

By immunohistochemistry on A204 cell culture slides, the 1E8.33 mAb showed a strong cytoplasmic granular staining pattern (Fig. 3A), similar to the previously described for the rabbit polyclonal antiserum to human procollagen 11A1 on tumor-associated myofibroblasts (21). The extent of this staining was lesser on SAOS-2 cells (Fig. 3C); and, on T98G cells, it was faint and reduced to a few granules in close contact with nuclei (Fig. 3E).

The staining pattern with anti-COL V on A204 and on SAOS-2 cells was the opposite to the above-described for the 1E8.33 mAb (Fig. 3B and D, respectively). The T98G cells were rather slightly stained with the anti-COL V polyclonal preparation (Fig. 3F). To validate these observations, we measured the amounts of COL11A1 and COL5A1 mRNA expressed by these cell lines, and performed Western blot analyses with the 1E8.33 mAb and anti-COL V on cell lysates.

A204 cells express high levels of COL11A1 mRNA, as shown by quantitative RT-PCR (Table III), with an SLR of 12.65, relative to the expression of this mRNA in normal breast samples; the SLR for COL5A1 was -0.65. SAOS-2 cells express lower levels of COL11A1 and higher COL5A1; and T98G cells, no COL11A1 and low COL5A1.

Table III. Relative gene expression of COL11A1 and COL5A1 in human cell lines with reference to normal breast tissues.

	SLR	
Cell line	COL11A1	COL5A1
A204	12.65	-0.65
SAOS-2	8.63	2.28
T98G	-5.78	0.53

By Western blotting, three major bands, of MW ~200, 120 and 97 kDa, were developed (Fig. 3G) by the 1E8.33 mAb on the A204 cell lysate, altogether with some less intense lower molecular weight bands; on lysates of both SAOS-2 and T98G cells, these bands were not apparent. When Western blot analyses were developed with the anti-COL V polyclonal preparation, a single band, of MW ~200 kDa, was seen on the three cell lysates (Fig. 3H); the signal on the SAOS-2 cell lysate was the strongest.

Therefore, we are able to show a high correlation in the expression of human proCOL11A1 in A204, SAOS-2 and T98G cells when investigated by means of three different methodologies such as immunohistochemistry, quantitative RT-PCR and Western blotting.

Epitope mapping of the 1E8.33 mAb. When the 1E8.33 mAb was probed on the above described multipeptide membrane, three consecutive spots (peptides 17-19: VDDFQEYNYGTMESY, FQEYNYGTMESYQTE and YNYGTMESYQTEAPR, respectively), were developed; the strongest signal was observed with peptide no. 19. According to these observations, the epitope recognized by the 1E8.33 mAb would encompass the YNYGTMESY amino acid sequence; the absolute absence of reactivity on the spot number 20 would imply an immunodominance for the YNY triad.

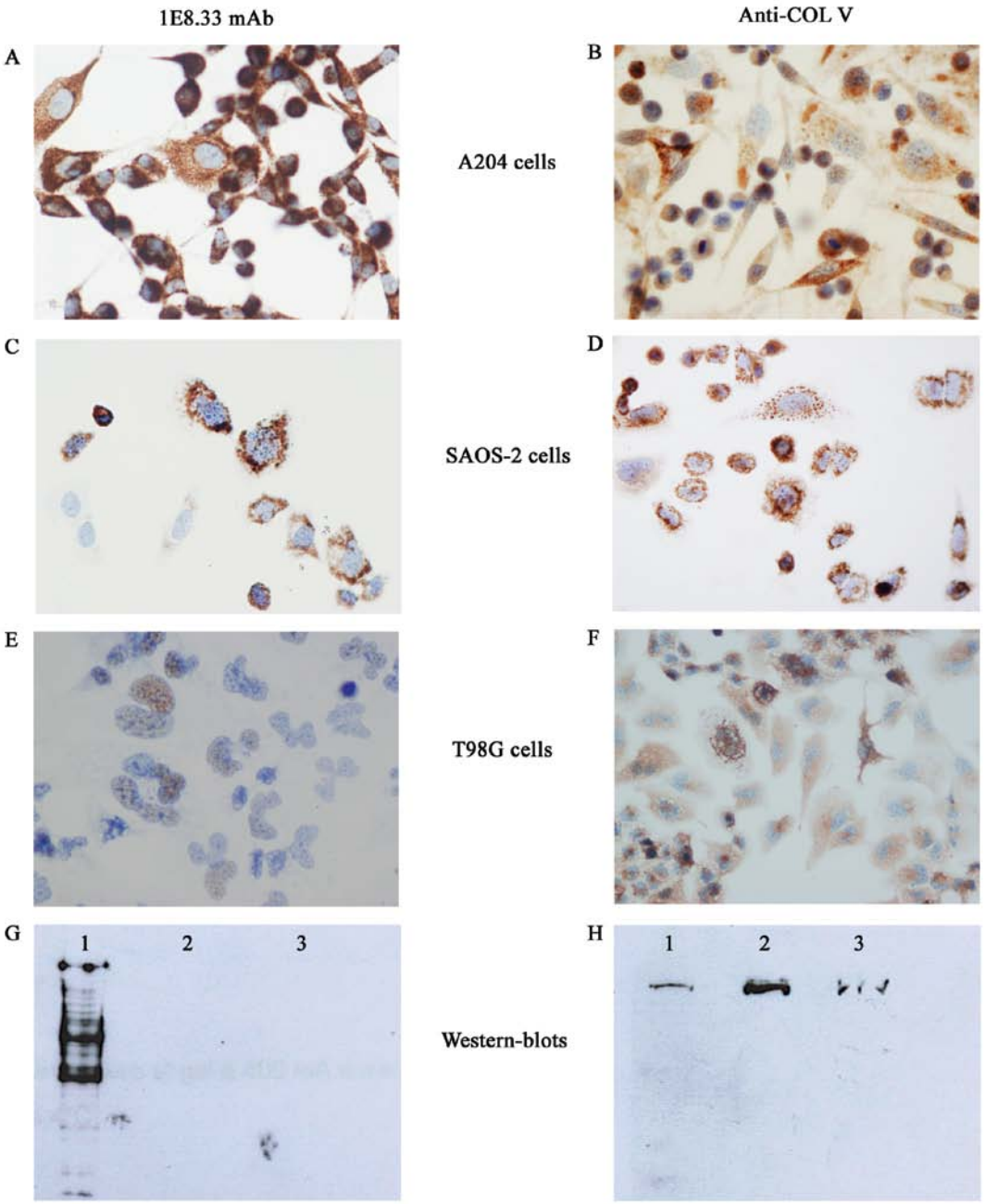

Figure 3. Cultured A204 cells (A) show a strong intracellular granular staining with the 1E8.33 mAb; this staining is less intense in cultured SAOS-2 cells (C) whereas cultured glioblastoma multiforme T98G cells (E) are hardly stained. By contrast, cultured SAOS-2 cells stain intensely with anti-COLV (D); this staining is lower in A204 and T98G cells (B and F, respectively) (original magnification x400). (G and H) Western blots with the 1E8.33 mAb. Lane 1, A204 cell lysate. Lane 2, SAOS-2 cell lysate. Lane 3, T98G cell lysate.

Then, a soluble N-acetylated and amido-ended 15 amino acid-long (311)YNYGTMESYQTEAPR(325) peptide was synthesized. This peptide was found to be blocking in Western blot assays developed on A204 cell lysates, while its homologous N-acetylated and amido-ended YDYVPSEDYYTPSPY peptide of human procollagen 5A1 was not blocking at all.

To finely assess the nature of the epitope, a series of shorter peptides: YNYGTMESYQTE, FQEYNYGTMES, FQEYNYG, YNYGTMESY, YDYVPSEDY, YNYGTMES, YNYGTME, YDYVPSE and YQTEAPR, were synthesized. Through similar inhibition tests, none of these tested peptides were found to be blockers; Fig. 4 shows a representative assay of this kind. These later findings suggest that, besides the core YNYGTMESY sequence, some of the QTEAPR neighbouring residues and, in particular, of the APR triad significantly contribute to antibody recognition. On the other hand, after examining available databases, the YNYGTMESYQTEAPR amino acid sequence is not recognizable in human proteins apart from the variable region of procollagen 11A1.

Altogether, these observations indicate that the 1E8.33 mAb recognizes an epitope in the YNYGTMESYQTEAPR sequence, only present in procollagen 11A1 among human proteins, and therefore we state that this mAb allows the specific identification of this cellular component in human tissues.

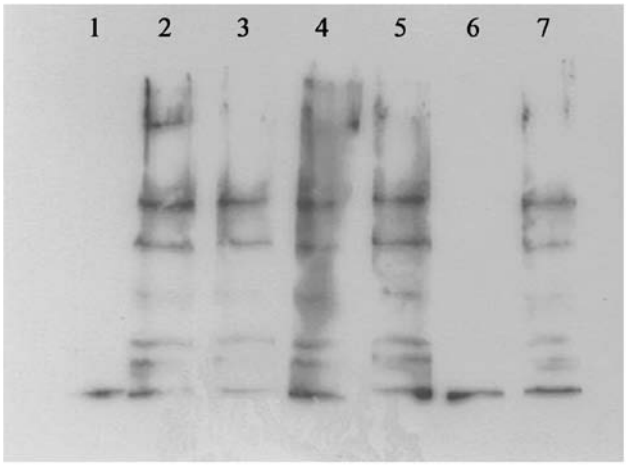

Figure 4. Representative Western blot inhibition tests with soluble peptides on an A204 cell lysate. Lane 1, negative control with only Dako Diluent and no 1E8.33 mAb. Lanes 2-6, mixtures of the 1E8.33 mAb incubated with FQEYNYG, YNYGTMES, FQEYNYGTMES, YNYGTMESYQTE and YNYGTMESYQTEAPR peptides, respectively. Lane 7, positive control with the 1E8.33 mAb in Dako Diluent. Only the YNYGTMESYQTEAPR peptide showed blocking capacity of the recognition of human procollagen 11A1 by the 1E8.33 mAb.

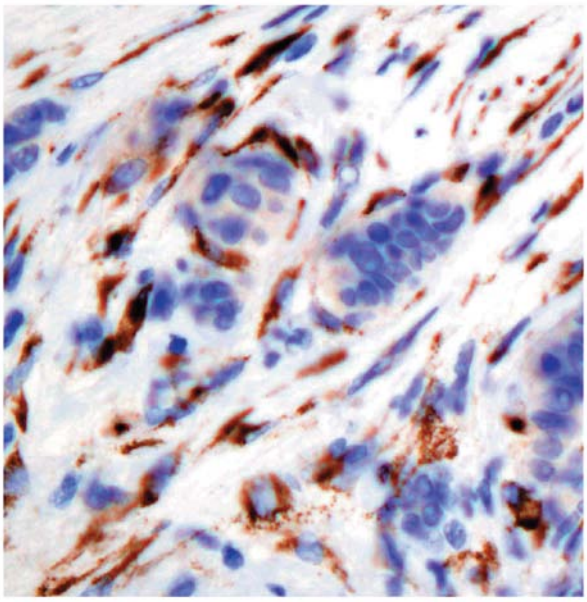

Figure 6. Representative staining of breast tumor (biopsy no. 10B7883) with the 1E8.33 mAb. Peritumoral myofibroblasts show a strong intracellular staining (original magnification x400).

Examination of human breast tissues. To further validate the performance of this mAb, 10 human breast tumor samples, selected for their positive staining with the 1E8.33 mAb, and their normal breast counterparts were examined, as above described for human cell lines, by quantitative RT-PCR in relation to the expression of COL11A1 and COL5A1 mRNA.

Breast tumor biopsies overexpressed COL11A1 mRNA (Fig. 5) and correlatively showed a strong cytoplasmic staining of peritumoral myofibroblasts with the 1E8.33 mAb (Fig. 6); the expression of COL5A1 mRNA was much lower than that of COL11A. Normal breast tissues were not stained with the 1E8.33 mAb.

When IHC blocking assays with the above-mentioned soluble peptides were performed on 10B7883 tumor biopsy slides, only the YNYGTMESYQTEAPR peptide blocked the staining of the 1E8.33 mAb in accordance with the above described Western blot inhibition tests.

Discussion

The COL5A1 and COL11A1 genes encode for the α1 chain of procollagens of type V and XI, respectively, which are extracellular matrix minor fibrillar collagens. These genes have been shown to be overexpressed in the stroma associated to some human tumors and in mesenchymal-derived tumor cell lines; this confers to these procollagens a significant interest as markers of tumor development. The availability of immunological tools, such as antibodies, highly specific for each one, may be very helpful for diagnosis and/or tumor evaluation.

This work described the characteristics of a novel mouse mAb which is highly specific for human procollagen 11A1. The epitope recognized by this mAb is encompassed in the YNYGTMESYQTEAPR sequence which, among human proteins, is only found in the variable region of procol-

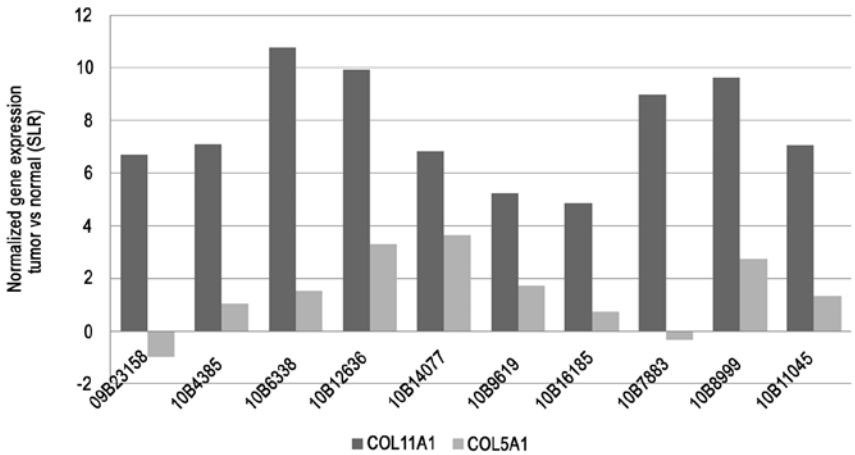

Figure 5. Q-RT-PCR data of COL11A1 and COL5A1 expression in breast tumor biopsies relative to their normal counterparts.

lagen 11A1. The 133 amino acid stretch of human procollagen 11A1 which was used for immunization and rescue of the 1E8.33 mAb shares 81% identities with the mouse homologous one within the variable region of COL11A1; even more, most of the above-mentioned epitope sequence is also present in the mouse homologous protein (YNYGTMEPYQTETPR). This high degree of sequence identity and the negative selection imposed on autoreactive B-cell clones may explain why the VH and VL domains of the 1E8.33 mAb are poorly mutated; some other similar human collagen-specific mouse mAbs have also been reported as being encoded by germ line configurations (43).

Mostly only polyclonal antibody preparations to human procollagen 11A1 have been described and/or are commercially available. The generation of some monoclonals from synthetic amino acid stretches of procollagen 11A1 of various species has been reported, but these monoclonal antibodies have not been characterized in detail (44,45). Thus, to our knowledge, we presently describe, for the first time, the characteristics and epitope amino acid sequence of a novel mouse monoclonal antibody specific for human procollagen 11A1.

We provide a series of evidence showing high correlation among them. In accordance with quantitative RT-PCR measurements, this antibody allows the immunodetection of human procollagen 11A1 by Western blotting and immunohistochemistry in human tumor cell lines and breast tumors. It develops a strong intracytoplasmic staining, an observation which is in agreement with the intracellular location of procollagens.

We have previously reported that COL11A1 mRNA is significantly overexpressed in pancreatic ductal adenocarcinoma in relation to chronic pancreatitis, and shown that a rabbit polyclonal antiserum to the variable region of human COL11A1 allows to immunohistochemically differentiate pancreatic cancer from chronic pancreatitis (21). Similarly, this polyclonal antiserum has been shown to be useful for differential diagnosis between benign sclerosing adenosis and malignant lesions of the breast (28). We now provide evidence that the 1E8.33 mAb allows also to identify the expression of procollagen 11A1 in the stromal myofibroblasts of human breast tumors. As COL11A1 has been reported to be overexpressed in some other tumors, the 1E8.33 mAb seems to be a very valuable diagnostic tool to characterize and evaluate human tumors with desmoplastic reaction.

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