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Recombinant and tissue-derived mouse BM-40 bind to several collagen types and have increased affinities after proteolytic activation

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Abstract. The calcium-binding extracellular matrix protein BM-40 was obtained as a mouse cDNA product from a stably transfected kidney cell clone. Electrophoresis and N-terminal sequence analysis demonstrated absence of the proteolytic processing previously observed for a mouse tumour-derived BM-40. Yet the two forms of BM-40 were very similar in their CD spectra, their calcium-dependent change in α helix content and their immunological epitopes. In surface plasmon resonance assays, recombinant mouse BM-40 showed distinct binding to the triple-helical domains of collagens I, II, III, IV and V with $K_d = 1–4$ μM but no binding to collagen VI. These interactions were abolished in the presence of EDTA. Tissue-derived mouse BM-40, however, bound collagens I and IV with $K_d = 0.1–0.2$ μM. Activation of collagen binding to give a similar K_d could be achieved for recombinant mouse BM-40 by treatment with the matrix metalloproteinase collagenase-3. The major cleavage site was located in helix C of the extracellular calcium-binding module of BM-40 and other less prominent cleavages occurred close to the N-terminus. The sensitive helix C site was just one residue away from that sensitive to endogenous tissue proteolysis, suggesting that cleavage could be a physiological mechanism to modulate collagen binding.

Key words. Calcium binding; collagen affinity; extracellular matrix; kinetic analysis; matrix metalloproteinase.

Abbreviations. CD = circular dichroism, EC module = extracellular calcium-binding module, FS module = follistatin-like module, MMP = matrix metalloproteinase, PCR = polymerase chain reaction.

A small glycoprotein of 33 kDa, known as SPARC, BM-40 or osteonectin, has been identified in bone, basement membranes and several other extracellular tissues and is produced by a large variety of cells [1, 2]. It shows high affinity for calcium [3] and calcium-dependent binding to various collagens [4–8]. Even though the exact biological role of BM-40 is still unknown, it is expressed at a high level during tissue repair and differentiation, where it may act as an anti-adhesive factor for cells [1, 2]. The most recent determination of the BM-40 structure indicates the presence of three distinct domains [9, 10]. They include an acidic N-terminal domain I, a central follistatin-like (FS) domain and a C-terminal extracellular calcium-binding (EC) domain. The latter is responsible for high affinity calcium binding through a pair of EF hands [10] and also possesses a collagen-binding epitope [9, 11]. Adjacent FS and EC modules have also been detected in the cDNA sequences encoding several other extracellular proteins including the proteoglycan testican [12]. This indicates the existence of a large family of related proteins [9, 10], although the other members of this family have not yet been characterized at the protein level.

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There is much evidence that the BM-40 structure and probably also its function can be modulated by extracellular proteolytic processing. This processing is fairly limited, yielding predominantly 30 and 10 kDa fragments, and occurs spontaneously in serum-free medium of cultured calvarial cells [13]. Osteoclastic cathepsin K produces a similar pattern of fragments and is thought to play a major role during bone resorption [14]. Partial degradation of BM-40 has also been observed with a bone-derived metalloproteinase that has not yet been characterized, and this degradation is accompanied by enhanced binding to collagen I [15]. A single cleavage within the EC module has been identified in BM-40 isolated from a mouse tumour basement membrane [16]. This tissue-derived mouse BM-40 had a 10-fold higher affinity for mouse and human collagen IV than recombinant human BM-40 [9], suggesting that proteolysis could be a mechanism to modulate collagen binding in situ.

In the present study we have examined this possibility by producing recombinant mouse BM-40 in an uncleaved form. This recombinant product bound with moderate and comparable affinities to collagens I to V but not to collagen VI. Limited cleavage by a matrix metalloproteinase (MMP-13) enhanced collagen binding to a level similar to that observed for tumour-derived mouse BM-40.

Materials and methods

Sources of proteins and proteases. Fibrillar collagens I, III and V were solubilized from human placenta by pepsin digestion and separated from one another by fractional NaCl precipitation [17]. Collagen IV was obtained from a similar digest as previously described [18]. The triple-helical domain of collagen VI was also extracted from human placenta by pepsin digestion and purified following an earlier protocol [19]. Cartilage collagen II was obtained as recombinant product [20] and kindly provided by D. J. Prockop. The Engelbreth-Holm-Swarm mouse tumour was used to purify a tissue-derived form of BM-40 [6, 16]. Recombinant human BM-40 has been described previously [7]. Collagenase-3 (MMP-13), prepared by a recombinant procedure [21], was a gift of G. Murphy. It was used at an enzyme-substrate ratio of 1:10 for cleavage of BM-40 in the presence of 2 mM $CaCl_2$ (24 h, 37°) and the reaction was stopped by adding 4 mM EDTA [11].

Construction of expression vector and purification of recombinant mouse BM-40. Total RNA (5 μg) from mouse 3T3 cells was reverse transcribed in a volume of 30 μl using Superscript reverse transcriptase as recommended by the manufacturer (Gibco BRL) and the oligonucleotide 5′-GGCGGAACAGCCAACCATCC, which is located in the 3′ untranslated region of the BM-40 cDNA. 10 μl of the reaction product was used to amplify the complete coding region by polymerase chain reaction (PCR) using the 5′ primer 5′-TTCCTGCAGCCCTTCAGACC and the 3′ primer 5′-GGTAATGGGAGGGGTGACAC. These primers amplify the sequence from nucleotide positions 4 to 1060 of the mouse cDNA [22]. PCR cycling conditions were 94 °C for 5 min, followed by 94 °C for 45 s, 52 °C for 45 s and 72 °C for 45 s (30 cycles). The PCR fragment was then digested with *Pst*I using endogenous restriction sites and subcloned into the *Pst*I-digested pBluescript KS(+) (Stratagene). The PCR fragment was verified by DNA sequencing. A *Not*I/*Cla*I-fragment containing the entire BM-40 cDNA was isolated from the Bluescript vector and inserted in the NotI/ClaI-restricted eukaryotic expression vector pCis [23].

Human embryonic 293 kidney cells were cotransfected with the expression vector and plasmid pSV_2pac, and stably transfected clones were selected with puromycin. Those producing large amounts of recombinant BM-40 were then identified by SDS gel electrophoresis [7]. A single clone was used to produce larger amounts of serum-free culture medium (100–200 ml) in a hollow fibre system [24]. This medium was used for the purification of BM-40 by chromatography on DEAE cellulose and Superose 12 [6]. Where necessary, further purification was achieved on a MonoQ column [7] or by reverse phase chromatography on a C_{18} column [6].

Surface plasmon resonance assay. Surface plasmon resonance binding studies were performed with BIAcore instrumentation (BIAcore AB, Uppsala). Collagens were immobilized by covalent coupling to CM5 sensor chips (BIAcore AB). After activation of the carboxymethylated dextran layer of the chip by addition of 35 μl of a mixture of 0.05 M N-hydroxysuccinimide and 0.2 M N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide, 60 μl of a solutioof 200 μg/ml collagen in 0.5 M sodium acetate pH 4.0 was added at a flow rate of 5 μl/min. The residual activated carboxylic groups of the chip were subsequently blocked by reaction with 35 μl of 1 M ethanolamine, adjusted to pH 8.5. These immobilization reactions resulted in 5000–6000 resonance units, equivalent to about 5–6 ng/mm^2 of immobilized collagens. Binding assays were performed in neutral buffer containing 0.05% P20 surfactant [9] and 2 mM $CaCl_2$ or 4 mM EDTA at a flow rate of 20 μl/min. BM-40 proteins were applied as soluble ligands at concentrations of 3 to 20 μM. These assays resulted in signals of 30–120 resonance units. Kinetic rate constants were calculated by nonlinear fitting of the binding and the dissociation curves by BIAevaluation software version 2.1 supplied by the manufacturer, according to [25].

Circular dichroism (CD) spectra. Samples of BM-40 were dialyzed against 5 mM Tris-HCl, pH 7.4, and their CD spectra in the far UV region were recorded at 25 °C on a JASCO 715 CD spectropolarimeter in a thermostatted quartz cell with an optical pathlength of 1 mm. Spectra were measured first in the presence of 2 mM $CaCl_2$ and then again after adding 6 mM EDTA. Molar ellipticities [θ] were calculated assuming a mean residue molecular mass of 110 Da and were expressed in deg cm^2 dmol^{-1}.

Analytical methods. Amino acid compositions and protein concentrations were determined on a LC3000 analyser (Biotronik) after hydrolysis with 6 M HCl (16 h, 110 °C). SDS polyacrylamide gel electrophoresis in 10–20% gradient gels followed standard procedures.

Table 1. Amino-terminal amino acid sequences of recombinant BM-40 and several of its proteolytic fragments.

Component	Position number: Sequence
Recombinant BM-40	1: APQQTEVAEEIV
32 kDa; band a; r, t	1: APQQTEVAEE
28 kDa; band b; r, t	30: VQVEMGEFED
	32: VEMGEFEDGA(m)
10 kDa; band c; r	197: LLARDFEKNY
10 kDa; band c; t	197: LLARDFEKNY
	198: LARDFEKNYN
	199: ARDFEKNYNM(m)

Proteolytic fragments are referred to by their apparent molecular mass (in kDa), the band identification (see fig. 1) and their origin from recombinant (r) or tissue-derived (t) BM-40. (m) indicates a minor component as judged from the yields by Edman degradation. Position numbering was according to [27].

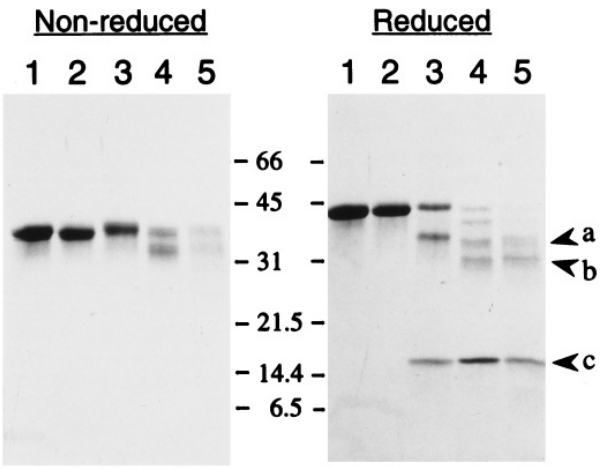

Figure 1. Comparison of recombinant and tissue-derived BM-40 and of its collagenase-3 digests by SDS gel electrophoresis. Lanes 1: recombinant human BM-40; lanes 2: recombinant mouse BM-40; lanes 3: tissue-derived mouse BM-40; lanes 4: digest of recombinant mouse BM-40; lanes 5: digest of tissue-derived mouse BM-40. Protein bands a, b and c were electroblotted and subjected to Edman degradation (table 1). Gels were calibrated with either nonreduced or reduced marker proteins and their positions are indicated in kDa.

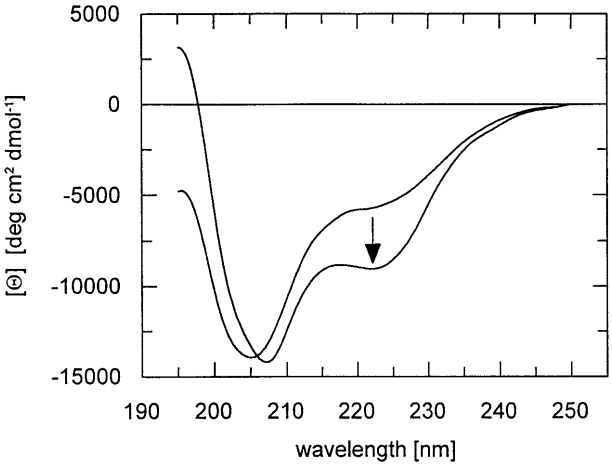

Figure 2. Conformational analysis of recombinant mouse BM-40 by CD spectroscopy. Spectra were recorded in buffer containing either 2 mM $CaCl_2$ or 4 mM EDTA. The arrow indicates the 38% decrease of ellipticity at 222 nm upon addition of calcium, indicative of an increase in α-helical content.

Electrophoretically separated protease digests were blotted onto Immobilon PSQ membranes (Millipore) and individual bands were excised and used for N-terminal sequencing on a Procise sequencer (Applied Biosystems) following the instructions of the manufacturer. ELISAs and immunoblotting [26] followed standard protocols.

Results

Structural comparison of recombinant and tissue-derived mouse BM-40. Since tissue-derived BM-40 from a mouse tumour showed substantial cleavage (up to 50%) at a single peptide bond within a large disulphide-bonded loop of its EC module [16], we used an established procedure [7] to obtain intact mouse BM-40 in recombinant form from a transfected human cell clone. The purified recombinant mouse BM-40 showed a single electrophoretic band prior to and after reduction (about 40 kDa) and was thus indistinguishable from recombinant human BM-40 (fig. 1, lanes 1 and 2). This is in clear contrast to the tumour-derived BM-40, which showed three bands after reduction, including two fragments of about 32 kDa and 10 kDa (fig. 1, lane 3). These fragments were previously shown [16] to contain the N-terminal sequence (32 kDa) and a sequence LARDFEKNYN starting at position 198 (10 kDa). In contrast, recombinant mouse BM-40 sequenced without prior electrophoresis showed the expected N-terminal sequence APQQTEVA and smaller amounts of a sequence lacking the most N-terminal alanine (table 1). Amino acid analysis of recombinant mouse BM-40 demonstrated, within the limits of analytical error, the composition predicted from the cDNA sequence (data not shown). This indicated that the recombinant protein was obtained in an essentially nondegraded form.

The formation of internal disulphide bonds in recombinant mouse BM-40 was demonstrated by a shift of the electrophoretic band to an apparently higher molecular mass after reduction (fig. 1, lanes 2). Furthermore, CD spectra of the recombinant mouse BM-40 were characteristic for a protein with high α-helical content and were indistinguishable from the spectra of recombinant human BM-40 [7]. A large conformational change upon removal of calcium was revealed by a 38% increase in molar ellipticity at 222 nm (fig. 2). This change was completely reversible upon re-addition of excess calcium (data not shown). The α-helical content, as judged from the ellipticity at 222 nm, of tissue-derived BM-40 [3, 6] compared to recombinant mouse BM-40 was slightly reduced (by about 10%) in the presence of calcium but was identical in its absence. The data are consistent with a native conformation of the recombinant protein and an only partial unfolding of the cleaved helix within the EC domain in the tissue-derived material.

An antiserum raised against tissue-derived mouse BM-40 was previously shown to react mainly with conformation-dependent epitopes [6]. This antiserum was now shown by ELISA titration to react equally well with tissue-derived and recombinant mouse BM-40 but not with human BM-40 (fig. 3A). However, an antiserum against recombinant human BM-40 showed a distinct crossreaction with both forms of mouse BM-40 (fig. 3B). Together, these data demonstrate that recombinant mouse BM-40 was obtained in native, undegraded form.

Identification of proteolytic cleavage sites. Recent studies with human BM-40 [11] have shown that several matrix metalloproteinases mimic the endogenous

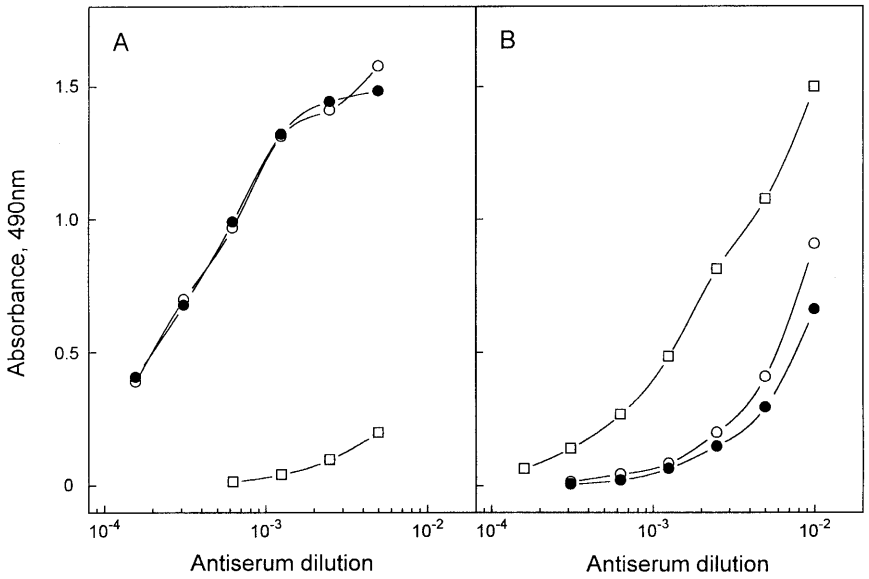

Figure 3. Immunological comparison of recombinant and tissue-derived mouse and human BM-40 by ELISA titration. Rabbit antisera used were either against tissue-derived mouse BM-40 (*A*) or against recombinant human BM-40 (*B*). Wells were coated with recombinant mouse BM-40 (○), tissue-derived mouse BM-40 (●) or recombinant human BM-40 (□).

cleavage observed with tissue-derived mouse BM-40 (see above). We have now cleaved recombinant mouse BM-40 with collagenase-3 (MMP-13), which led to substantial conversion into 32, 28 and 10 kDa fragments after reduction (fig. 1, lane 4). Sequence analysis demonstrated that the 32 kDa band had the BM-40 N-terminal sequence and the 10 kDa band had a single sequence LLARD starting at position 197. These two fragments apparently account for the whole of BM-40, indicating a major cleavage site at peptide bond 196–197, with the fragments held together by a disulphide bridge [6, 16]. N-terminal sequencing of the 28 kDa band demonstrated two more cleavage sites within the N-terminal domain I (table 1). Cleavage of tissue-derived mouse BM-40 with MMP-13 caused a complete conversion of the original 42 kDa band into the same set of fragments seen with recombinant mouse BM-40 after reduction (fig. 1, lane 5). The 32 and 28 kDa bands of both digests were identical in their N-terminal sequences. The 10 kDa band, however, showed two major N-terminal sequences in about equal proportions, one starting at position 197 and the other at position 198 (table 1). The latter was identical to that generated by endogenous proteolysis [16].

Binding to various collagen types in surface plasmon resonance assay. Previous studies have demonstrated that BM-40 binds to several collagen types but did not clarify whether this occurs with similar affinities and through identical BM-40 sites [4–9]. We have now used a kinetic analysis by surface plasmon resonance assay (fig. 4) in order to compare K_d values based on association and dissociation rate constants for the binding to collagen types I to VI (table 2). This reproducibly demonstrated apparently identical binding activities ($K_d = 1.3–1.75$ μM) of recombinant mouse BM-40 for human collagens I and IV. Comparable or slightly reduced affinities for collagens II, III and V were observed in single measurements, but no binding was observed with the triple-helical domain of collagen VI. All of these interactions were measured in the presence of 2 mM $CaCl_2$, and addition of 10 mM EDTA abolished binding. This indicated that the calcium-binding EC module of BM-40 is responsible for binding.

Previous studies have shown that tissue-derived mouse BM-40 bound with higher affinities than recombinant human BM-40 to both mouse and human collagen IV [9]. This approximately 10-fold higher affinity ($K_d = 0.16–0.19$ μM) was confirmed here for collagen IV (fig. 4) and also demonstrated for the binding to collagen I (table 2). This strongly implied that the endogenous proteolysis of tissue-derived mouse BM-40 was responsible for the increase in its affinity. This was examined by treatment of recombinant BM-40 with collagenase-3, which causes a similar cleavage and resulted in a 4- to 8-fold increase in the affinity of recombinant BM-40 for collagens I and IV. Distinctly smaller increases were observed after the same treatment of tissue-derived mouse BM-40 (table 2). This is very likely due to the fact that partial activation has already occurred through endogenous proteolysis.

Discussion

BM-40 was the first extracellular matrix protein shown to bind calcium with high affinity through two EF hands located in its EC module [3, 10, 28]. It is also a versatile collagen-binding protein [4–9, 29] and a major calcium-dependent binding epitope has been mapped to

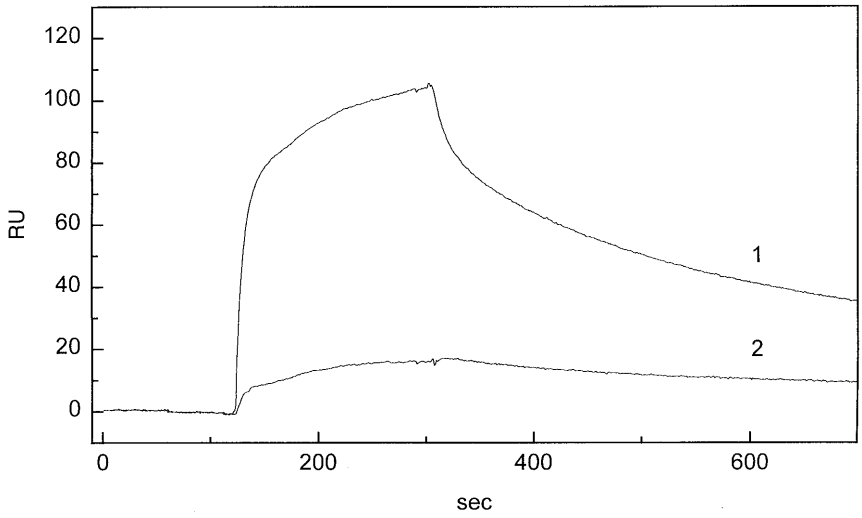

Figure 4. Binding profiles of two forms of mouse BM-40 to collagen IV in surface plasmon resonance assay. Curve 1: tissue-derived, curve 2: recombinant. Binding was started after 120 sec and dissociation at 300 sec. RU = resonance units.

the EC module [6, 9, 11, 28]. The present study started from a previously puzzling observation that tissue-derived mouse BM-40 bound to human and mouse collagen IV with about 10-fold higher affinity than did recombinant human BM-40 [9]. The BM-40 sequences of these two species have a high identity, their 149-residue EC modules differing by only five, mainly conservative, substitutions [27]. It was therefore not clear whether the small sequence differences or a substantial proteolytic cleavage at a single site in the EC module of tissue-derived mouse BM-40 [16] were responsible for the difference in affinity. In order to discriminate between these two possibilities, we have now generated intact recombinant mouse BM-40 and clearly show modulation of its binding affinity through proteolysis.

Recombinant mouse BM-40 was obtained in a native form as shown by disulphide bonding, CD spectroscopy and the presence of conformation-dependent antigenic epitopes. In a kinetic binding assay, it interacted with collagens I to V with comparable affinities ($K_d = 1–4$ μM), suggesting that the same or overlapping binding epitopes of the EC module are responsible for binding to each collagen type [9, 11]. Yet the triple-helical domain of the microfibrillar collagen VI [19] did not bind BM-40, which indicated that a triple-helical conformation per se is not sufficient for binding. The same range of affinities for several collagen types ($K_d = 2–6$ μM) was previously demonstrated for recombinant human BM-40 [9, 11], which eliminated the possibility that small sequence differences between the two species are responsible for the difference in affinity. This was underscored by the 5- to 10-fold increase in affinity for collagens I and IV observed after treatment of mouse (table 2) and human BM-40 [11] with collagenase-3. This suggests that similar mechanisms could operate in vivo and irreversibly change the affinity of BM-40 for other extracellular matrix ligands. Such processes are usually under the control of a cascade of interactions, in agreement with recent observations that collagenase-3 itself needs activation by other matrix metalloproteinases or plasmin [30].

From a recent crystallographic analysis of the EC module of BM-40 [10], it has now become clear that the adjacent peptide bonds cleaved by collagenase-3 and an undefined endogenous protease (table 1) are located in helix C of the three-dimensional structure. Helix C was shown to belong to the connecting region between the long N-terminal helix A and the pair of calcium-binding EF hands [10]. Collagenase-3 also cleaves two peptide bonds in the N-terminal domain I, although with lower rates, but no such cleavages are found in tissue-derived mouse BM-40 [16]. This demonstrated unequivocally that a single cleavage in helix C is responsible for the increase in collagen affinity. Furthermore, proteolytic activation is mainly due to an increase in the association rate constants for collagen binding (table 2), consistent with the interpretation that steric hindrance is responsible for the lower binding efficiency of uncleaved BM-40. This is also in agreement with the crystal structure, where helix C covers part of the surface of helix A [10], suggesting that the latter contributes the collagen binding epitope. This prediction was recently supported by site-directed mutagenesis of a single residue of helix A, which abolished collagen IV binding (T. Sasaki, R. Timpl, unpublished).

The physiological role of proteolytic activation of BM-40 could be to increase collagen binding in tissues where the concentrations of one or both ligands is distinctly below the binding potential of uncleaved BM-40 ($K_d = 1–4$ μM). There is already some evidence that proteolysis could occur in tissue and cell cultures [13–16, 31], although in most of these cases the involvement of helix C has not yet been demonstrated. Similar cleavages also

Table 2. Binding of recombinant and tissue-derived mouse BM-40 to various human collagen types and activation of binding by collagenase-3 treatment.

Collagen type	Source of BM-40	k_{diss} (s^{-1})	k_{ass} (M^{-1} s^{-1})	K_d (μM)
IV	recombinant	$1.4 \pm 0.2 \times 10^{-3}$	800 ± 400	1.75 ± 0.4
	tissue	$2.0 \pm 0.4 \times 10^{-3}$	10500 ± 2700	0.19 ± 0.04
	recombinant, +C	2.5×10^{-3}	6300	0.40
	tissue, +C	2.0×10^{-3}	22000	0.09
I	recombinant	$1.4 \pm 0.2 \times 10^{-3}$	1100 ± 500	1.3 ± 0.5
	tissue	$3.1 \pm 0.8 \times 10^{-3}$	35700 ± 1500	0.09 ± 0.03
	recombinant, +C	3.0×10^{-3}	19000	0.16
	tissue, +C	3.2×10^{-3}	31000	0.10
II	recombinant	3.2×10^{-3}	600	4.8
III	recombinant	1.5×10^{-3}	1200	1.3
V	recombinant	1.1×10^{-3}	260	4.4
VI	recombinant	no binding		

Kinetic measurements were performed by surface plasmon resonance assay using collagens immobilized on sensor chips and BM-40 as soluble ligand, in the concentration range 3–20 μM. Treatment with collagenase is indicated by +C. Several of the values are expressed as mean ±S.D. of 2–4 independent determinations.

occur in melanomas and were correlated with neoplastic progression [32, 33]. Based on our present data, it would seem worthwhile to generate antibodies against immunological neo-epitopes at the novel N-terminal site of helix C (i.e. against synthetic peptides LLARD and LARD). Such an approach is likely to be feasible, as shown for matrix metalloproteinase cleavage sites in other extracellular substrates such as aggrecan [34], and would allow examination of a large number of tissues either as immunohistological sections or in the form of small extracts. The widespread expression of BM-40, particularly during tissue remodelling such as that occurring at many developmental stages, wound repair and tumour growth [2], would make it an appropriate model for such studies.

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