Immunochemical distinction between two different chains of type IV collagen

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Antisera against mouse and human basement-membrane type IV collagen showed in radioimmunoassays distinct binding with large pepsin fragments obtained from the C-terminal portions of $\alpha 1(\text{IV})$ - and $\alpha 2(\text{IV})$ -chains. These reactions were specific for each constituent polypeptide chain. The data were confirmed by immunoabsorption, allowing the separation of antibodies with restricted chain specificity. Inhibition assays with CNBr peptides demonstrated the different localizations of antigenic determinants, which were either species-specific or shared by the human and mouse antigens.

Previous studies have suggested that basement-membrane (type IV) collagen is composed of a single species of α -chain tentatively named $\alpha 1(\text{IV})$ (Kefalides, 1971; Dehm & Kefalides, 1978). More recent analyses of pepsin fragments of type IV collagen obtained from a mouse tumour (Timpl *et al.*, 1979a), human placenta (Bailey *et al.*, 1979; Glanville *et al.*, 1979; Kresina & Miller, 1979; Sage *et al.*, 1979), human kidney (Daniels & Chu, 1975; Dixit, 1979) and bovine lens capsule (Dixit & Kang, 1979; Gay & Miller, 1979) have indicated a more complex nature of the constituent polypeptide chains. The data showed two sets of fragments basically different in amino acid composition, CNBr-peptide patterns and amino acid sequences. These two sets of fragments included small variants ($M_r = 48\,000$ – $55\,000$) named P1 and P2 (Timpl *et al.*, 1979a) or $50K_1$ and $50K_2$ (Kresina & Miller, 1979; Dixit, 1979) or larger chains ($M_r = 70\,000$ – $95\,000$) named $\alpha(\text{IV})$ and $\alpha(\text{IV})'$ (Glanville *et al.*, 1979) or C and D (Kresina & Miller, 1979; Gay & Miller, 1979; Dixit, 1979; Dixit & Kang, 1979). Although these fragments comprise only a portion of the original chains of type IV collagen, which may have a size of $M_r = 140\,000$ – $160\,000$ (Timpl *et al.*, 1978), they were considered to be derived from two structurally different polypeptides. A similar heterogeneity was also found in biosynthetic studies (Crouch *et al.*, 1980; Tryggvason *et al.*, 1980; Alitalo *et al.*, 1980), demonstrating two chains ($M_r = 165\,000$ – $185\,000$) in the precursor form of type IV collagen. Following a suggestion of Crouch *et al.* (1980), we have named the two constituent polypeptide chains of type IV collagen as $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ in accordance with the nomenclature used for interstitial collagens, but have maintained

the previous use of the terms P1, P2, α and α' for smaller fragments of these constituent chains.

At present there is no clear evidence available as to whether these chains are assembled in different triple-helical molecules, even though indirect data (Bailey *et al.*, 1979; Timpl *et al.*, 1979a) favour this interpretation. We have recently described specific antibodies to native type IV collagen capable of reacting with unfolded polypeptide chains as shown by radioimmunoassay (Timpl *et al.*, 1979b). Since such antibodies may be useful for examining the composition of type IV collagen molecules, we have now studied the immunological reactions of well-characterized pepsin fragments.

Materials and methods

Preparation of pepsin fragments from type IV collagen

Fragments P1 and P2 were obtained from a mouse tumour basement membrane and purified by chromatography under denaturing or non-denaturing conditions (Timpl *et al.*, 1979a,c). The corresponding fragments α and α' were purified from a pepsin digest of human placenta as described previously (Glanville *et al.*, 1979). Each of these peptide chains showed a homogeneity of >90% when examined by electrophoresis in the presence of sodium dodecyl sulphate.

Isolation of CNBr peptides from fragment P1

Peptide P1 was reduced with mercaptoethanol in order to eliminate oxidized methionine (Adelstein & Kuehl, 1970), dissolved in 70% (v/v) formic acid (10 mg/ml) and cleaved with CNBr (20 mg/ml) for 4 h at 30°C. The resulting peptides were then

purified by chromatography on Sephadex G-75 and by ion-exchange chromatography (Schuppan, 1979; Schuppan *et al.*, 1980). The data showed the presence of three large peptides, CB1, CB2 ($M_r = 14000$) and CB3 ($M_r = 9000$) and, in addition, six smaller peptides ($M_r = 300-5000$). Each peptide was identified by a unique *N*-terminal amino acid sequence (Schuppan, 1979; Schuppan *et al.*, 1980).

Antisera and purification of antibodies

The rabbit antisera produced against acid-soluble type IV collagen from mouse tumour and against pepsin-solubilized type IV collagen from human placenta were those described previously (Timpl *et al.*, 1979b). A similar immunization schedule was followed in an attempt to produce antibodies against a mixture of fragments P1 and P2 isolated in their triple-helical state (Timpl *et al.*, 1979a).

For immunoabsorption, 1g of CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) was briefly washed with 1M-HCl, suspended in a solution of denatured peptide P1 or P2 (15 mg/20 ml of 0.2M-phosphate, pH 8.0) and stirred for 3 days at 6–8°C. The coupling reaction was terminated by incubating the gels in 20 ml of 0.1M-Tris (1 h at room temperature), followed by excessive washing with phosphate-buffered saline, pH 7.2 (0.12M-NaCl/0.16M-sodium phosphate/0.003M-sodium azide), 0.1M-acetic acid, 0.05M-HCl/0.15M-NaCl and phosphate-buffered saline again. Isolation of antibody from 20–30 ml of pooled antisera against mouse or human type IV collagen followed a previously used schedule (Beil *et al.*, 1973) and included two passages over the P1 adsorbent, followed by two passages over the P2 adsorbent. Antibodies displaced from the P1 adsorbent were finally passed over the P2 adsorbent and vice versa in order to eliminate cross-reacting antibodies.

Radioimmunoassays

Labelling of the peptides with Na^{125}I by the chloramine-T method (McConahey & Dixon, 1966) or with ^{125}I -labelled Bolton–Hunter reagent (iodo-*p*-hydroxyphenylpropionic acid *N*-hydroxy-succinimide ester) followed established procedures (Timpl *et al.*, 1979b; Tanswell *et al.*, 1978). Free reagent was removed from protein-bound radioactivity on a column (1 cm $\times$ 15 cm) of Bio-Gel P2 equilibrated in phosphate-buffered saline/0.04% Tween 20. About 70–80% of the label could be incorporated into the protein. Examination of peptides P1 and P2 labelled by the chloramine-T method using slab-gel electrophoresis (Laemmli, 1970) and autoradiography showed that most of the radioactivity migrated with authentic P1 or P2, in spite of a low tyrosine content in both peptides (Timpl *et al.*, 1979a).

Binding of labelled peptides by antisera and

inhibition of these reactions by non-labelled components by using them in an unfolded form were examined by a double-antibody assay (Timpl *et al.*, 1979b). All dilutions were done with phosphate-buffered saline/0.04% Tween 20/0.5% bovine serum albumin, in order to keep non-specific binding of labelled material to non-immune rabbit serum in the range 3–6%. During prolonged storage of labelled peptides there was an increase in non-specific binding, which could be blocked by adding an excess of non-labelled peptide. All inhibition data were corrected for this increase in non-specific binding (Walker & Keane, 1977).

Results

Binding of antibody to chain fragments of type IV collagen

We choose for this study peptides P1 ($M_r = 55000$) and P2 ($M_r = 48000$) from mouse type IV collagen and peptides α ($M_r = 95000$) and α' ($M_r = 70000$) from human type IV collagen. These peptides can be obtained in a sufficiently pure state (Timpl *et al.*, 1979a; Glanville *et al.*, 1979) suitable for radioimmunoassays, and, according to compositional data, apparently belong to either the $\alpha 1(\text{IV})$ -chain (P1 and α) or the $\alpha 2(\text{IV})$ -chain (P2 and α') of type IV collagen (for nomenclature, see the parenthetic note in the introduction). Several rabbit antisera raised against triple-helical mouse or human type IV collagen showed distinct binding to both labelled peptides P1 and P2 (Fig. 1). Binding

Fig. 1. Binding of ^{125}I -labelled fragments (a) P1 and (b) P2 from mouse type IV collagen $\alpha 1(\text{IV})$ - and $\alpha 2(\text{IV})$ -chain by rabbit antisera to mouse (●) and human (○) type IV collagen

With several antisera, maximal binding for P1 was in the range of 50–80% and for P2 in the range of 80–100%.

and inhibition data (see below) were the same regardless of whether P1 was labelled via tyrosine (chloramine-T oxidation) or via lysine residues (Bolton–Hunter reagent). The titre of binding P1 or P2 was usually stronger in the antisera against human type IV collagen than in the antisera against mouse type IV collagen. Much stronger binding still was observed if the antisera were made to react against triple-helical type IV collagens (Timpl *et al.*, 1979b). These data indicate that both fragments react only with a fraction of the total antibodies found in the antisera. Binding similar to that of P1 and P2 could be observed with the labelled fragments α and α' (results not shown). Antisera against human type IV collagen showed a significant reaction, whereas most of the antisera to mouse type IV collagen failed to react with α and α' .

Distinction between $\alpha 1(IV)$ - and $\alpha 2(IV)$ -chain fragments by inhibition assays

The binding of labelled P1 could be specifically inhibited by peptide P1, whereas about 50–100-fold greater amounts of peptide P2 were required to achieve the same result (Fig. 2a). Reverse-inhibition patterns were observed for the reaction of the same or other antisera with labelled P2 (Fig. 3a). An analogous relationship was found for the corresponding pair of fragments, α and α' , from human type IV collagen (Fig. 3b). These data indicated that the reaction with each fragment from the $\alpha 1(IV)$ - or $\alpha 2(IV)$ -chain is specific and apparently based on different populations of antibodies in the same individual antiserum. The low but distinct cross-reactions between both sets of fragments, e.g. P1 and P2 (Fig. 2a), may be explained by either a 100-fold

difference in affinity (Walker & Keane, 1977), or, more likely, by the possibility that each fragment may not be entirely pure but contaminated by about 1–2% of the other fragment.

The inhibition assays with several antisera also showed various extents of interspecies cross-reactions between corresponding mouse and human peptides. These cross-reactions were either high, as shown by almost identical inhibition curves (Fig. 3a), or only partial, as indicated by a different shape of the inhibition curve (Figs. 2a and 3b). Complete correspondence between mouse and human fragments was observed when the reaction of antisera against human type IV collagen with labelled mouse peptide P1 was examined by inhibition assays (Fig. 2b).

Localization of different antigenic determinants on CNBr peptides

The binding of labelled P1 by various antisera could also be inhibited by individual CNBr peptides (CB1–CB9) produced from P1. Peptide CB2 showed an inhibitory activity closely resembling that of fragment P1 when antisera against mouse type IV collagen were analysed (Fig. 4a). Other large peptides, such as CB1 and CB3, were, on a molar basis, about 10–20-fold weaker inhibitors. The binding of labelled P1 by antisera against human type IV collagen, however, could be efficiently blocked by peptide CB1, whereas peptides CB2 and CB3 showed almost no activity (Fig. 4b). Lack of substantial inhibition was also observed for the other, smaller, CNBr peptides (results not shown).

We also examined the reactions with labelled P2 and found both P2 and CNBr-cleaved P2 to be

Fig. 2. Inhibition assay for antibodies specific for fragments of the $\alpha 1(IV)$ -chain. Antisera against mouse (a) or human (b) type IV collagen were made to react against ^{125}I -labelled fragment P1 from the mouse. Inhibitors of the reactions were mouse P1 ($\square$), mouse P2 (Δ), human α ($\circ$) and human α' ($\bullet$).

Fig. 3. Inhibition assay for antibodies specific for fragments of $\alpha_2(\text{IV})$ -chain (a) Antiserum against mouse type IV collagen reacting with ^{125}I -labelled mouse P2. (b) Antiserum against human type IV collagen reacting with ^{125}I -labelled human α' . Inhibitors of these reactions were the same as in Fig. 2. The pattern shown in (a) is the exceptional rather than the usual observation. With other antisera much less cross-reaction was observed between P2 and α' .

Fig. 4. Localization of different antigenic determinants on mouse fragment P1 by inhibition assay with CNBr peptides. Antisera against mouse (a) or human (b) type IV collagen were made to react against ^{125}I -labelled P1. Inhibitors of the reactions were P1 (□) and the CNBr peptides CB1 (○), CB2 (●) and CB3 (△) derived from P1.

equivalent inhibitors. Localization of distinct antigenic determinants on individual CNBr peptides of P2 thus seems feasible.

Separation of antibodies specific for fragments P1 and P2

Initial attempts to raise antisera against fragments P1 and P2 by immunization with the fragments have

failed. We therefore tried to isolate by immuno-adsorption antibodies specific for P1 and P2 from antisera against triple-helical type IV collagen. Data obtained with an antiserum pool against the human antigen showed that such a separation could be accomplished and that the antibodies showed reasonable specificity in binding either labelled P1 or P2 (Fig. 5). A similar separation could be achieved

Fig. 5. Binding assays of antibodies purified from an antiserum to human type IV collagen by immuno-adsorption on mouse fragments P1 (a) and P2 (b). Test antigens were labelled fragments P1 (○) and P2 (●).

for antisera against the mouse antigen, but, as expected from binding data (Fig. 1), recovery of antibody activity was lower than that observed with antisera to human type IV collagen. We also found that some antibodies bound to both fragments P1 and P2, indicating shared antigenic structures.

Discussion

Chemical data have indicated that type IV collagen consists of at least two different polypeptide chains, $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$, which show sufficient structural differences to allow their separation at the level of large pepsin-digested fragments. Pepsin apparently cleaves within the triple-helical domain of the molecules (Schuppan *et al.*, 1980), giving rise to several fragments that may vary in size owing to variations in the conditions of proteolytic digestion or to interspecies differences in amino acid sequence (Bailey *et al.*, 1979; Dixit, 1979; Gay & Miller, 1979; Glanville *et al.*, 1979; Kresina & Miller, 1979; Sage *et al.*, 1979; Timpl *et al.*, 1979a). The precise order of the pepsin fragments within the polypeptide chains is not known, but recent data on CNBr peptides (Dixit & Kang, 1979; Schuppan *et al.*, 1980) indicate that the interchain disulphide bonds linking the triple helix are located at the *N*-terminus of the $\alpha 1(\text{IV})$ -chain, whereas the fragments named here, α and P1, originate from the *C*-terminal region. If structural homology exists in the $\alpha 2(\text{IV})$ -chain, then P2 should also represent the *C*-terminal portion of the chain, whereas fragment α' may contain P2 but with an additional extension at the *N*-terminus.

Our immunochemical data clearly support the chemical observations and showed distinct antigenic differences between the P1 and P2 fragments

from the mouse tumour and between the α and α' fragments from human placenta. The data also demonstrated a closer antigenic relationship between P1 and α than between P1 and P2, whereas the latter showed good cross-reactions with α' . These observations are in general agreement with the interpretations discussed above and have been extended recently by showing striking differences in CNBr peptides and *N*-terminal sequences for the P1 and P2 components (Timpl *et al.*, 1979a; Schuppan, 1979) as well as differences between α and α' from human placenta (Glanville *et al.*, 1979; A. Rauter & R. W. Glanville, unpublished work).

Previous studies have established that only some of the antibodies raised against mouse tumour type IV collagen cross-react with corresponding human collagens (Timpl *et al.*, 1979b; Wick *et al.*, 1979). Although we have occasionally observed that antisera to human type IV collagen may completely cross-react with mouse type IV collagen, the data presented here also show antigenic determinants specific for the human antigen. Even less antigenic similarity exists between $\alpha 1(\text{IV})$ - and $\alpha 2(\text{IV})$ -chain fragments when obtained from the same source. Similar relationships have been reported for different polypeptide chains of interstitial collagens (Timpl, 1976).

The major antigenic determinants of fragment P1 could be localized to CNBr peptide CB2, resembling about one quarter of the fragment (Fig. 4a). Based on other analyses (Fig. 2a) these sites show no or only little cross-reaction with human type IV collagen. The apparent restriction of the major antigenicity offers an approach to identify the primary structure of antigenic determinants in mouse type IV collagen such as previously carried out with other collagens (Timpl, 1976). Owing to the lack of CNBr peptides of fragments α and α' , it was not possible to localize major antigenic determinants of human type IV collagen. Minor antigenic determinants could be studied by using the cross-reaction between antisera to human type IV collagen and labelled fragment P1 from the mouse (see Fig. 2b). Here antigenicity was found entirely on CNBr peptide CB1 of fragment P1. It is to be expected that a similar structure exists on the human fragment α and that these antigenic determinants are structurally different from those localized on peptide CB2.

It was also possible to prepare chain-specific antibodies by immunoabsorption on P1 and P2 fragments. We showed previously that P1 and P2 could be separated from each other in a triple-helical state (Timpl *et al.*, 1979a), indicating that the mouse tumour primarily contains two species of type IV collagen, with the compositions $[\alpha 1(\text{IV})]_3$ and $[\alpha 2(\text{IV})]_3$. The antibodies specific for P1 and P2 may thus be useful for studying the composition of

type IV collagen from other sources, such as cell-derived products (Tryggvason *et al.*, 1980; Alitalo *et al.*, 1980), as well as the tissue distribution of such molecules by immunofluorescence (Wick *et al.*, 1979) and electron microscopy.

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