A New Form of Tumor and Fetal Collagen That Binds Laminin†

I. Pucci-Minafra,*,‡,§ C. Luparello,‡ M. Andriolo,‡ L. Basiricò,‡ A. Aquino,‡ and S. Minafra§,∥

Dipartimento di Biologia Cellulare e dello Sviluppo, Università, Via Archirafi 20, 90123 Palermo, Italy, Centro di Oncobiologia Sperimentale, Palermo, Italy, and Istituto di Istologia ed Embriologia, Università, Palermo, Italy

Received March 1, 1993; Revised Manuscript Received May 3, 1993

ABSTRACT: Human breast and colon carcinoma tissues contain a form of collagen, not described before, composed of α_1 chains of similar size (~100 kDa) but different charge. The three constitutive chains, separated by two-dimensional electrophoresis, are a unique acidic component, undetectable in other collagen types, with an apparent isoelectric point of 4–5, and two more basic components displaying the same electrophoretic behavior as α_1(III) and α_1(I), respectively. The acidic chain is structurally distinct from α_1(I) and displays a cyanogen bromide-derived fragment of similar size to CB5(III). This collagen in its native state is resistant to trypsin and metalloproteinase 3, while it is fully degraded by metalloproteinases 1 and 9. Moreover, this collagen appears able to bind to laminin, as tested by affinity chromatography. The biological significance of our data is related to the finding of this collagen form not only in the tumor tissue tested but also in embryonic–fetal tissues (bovine skin and intestine and human umbilical cord). For its peculiar laminin-binding ability and occurrence in tumoral and embryonic–fetal tissues, we propose to temporarily term this new collagen form OF/LB collagen (onco–fetal, laminin-binding collagen). The presence of OF/LB collagen during development and cancer, and its absence in normal adult tissues, make this protein a potential stromal marker of malignancy.

In the last few years a great deal of information has been produced in the field of collagen research, in relation to molecular genetics, protein structure, tissue distribution, and functional aspects. At present at least 14 different collagen types, comprising collectively 27 individual polypeptide chains, have been defined and classified according to a chronological nomenclature. The majority have been grouped into three subfamilies on the basis of their supramolecular structures and named: *fibril-forming collagens* (I, II, III, V, XI), *sheet-forming collagens* (IV, VIII), and *fibril-associated collagens with interrupted triple helices* (*FACITs*; IX, XII, XIV) [see Vuorio and De Crombrugghe (1990) and van der Rest and Garrone (1991) for reviews]. However, not all conform to these categories, since some collagen types can be present in tissues as homotrimeric or heterotrimeric molecules and also as hybrid molecules composed of mixed α chains of different collagen types [see references in van der Rest and Garrone (1991)].

The existence of such molecular variety within this protein family is the best indirect evidence for the multifunctional biological roles, at present still very enigmatic, played by different collagens within tissues. It is well known that during development and tissue remodeling the three-dimensional architecture of collagen and its associated components is of crucial importance for a number of cellular activities (Bornstein & Sage, 1989). Consequently, collagen disorders, either inherited or acquired, can lead to serious pathological conditions. A peculiar collagen disorder, still poorly understood, is that related to invasive solid cancers of "scirrhous" type, like breast and colon carcinomas.

We have previously reported that infiltrating ductal carcinoma (IDC) of the breast, one of the most aggressive of tumors, displays a profound disorganization of the collagenous stroma, as determined by both ultrastructural and biochemical studies (Pucci-Minafra et al., 1986, 1987, 1989). In particular, our analysis of collagen composition revealed the presence of an "unusual" form of collagen, absent in the normal counterpart (Luparello, 1987), which appeared to be composed of three identical α_1(I)-sized chains (Minafra et al., 1984; Pucci-Minafra et al., 1985; Luparello & Pucci-Minafra, 1986). This collagen was identified as "type I trimer" on the basis of an uninterrupted pepsin-resistant and collagenase-sensitive domain of M_r about 100 kDa, the absence of disulfide bonds, and the ability to form organized polymers in vitro. In addition, when used as a substrate for neoplastic cells in culture, this collagen exerted a different biological effect on cell behavior with respect to type I and V collagens (Schillaci et al., 1989; Luparello et al., 1990, 1991).

According to the literature, the so-called type I trimer is considered an embryonic–fetal collagen phenotype, in which the α_2 chain is substituted by a third identical α_1 chain (Miller & Gay, 1987). The information concerning the structural identity and tissue occurrence of type I trimer has been controversial and fragmentary, mainly due to the difficulties encountered in separating this molecule from regular type I collagen using conventional biochemical procedures. However, a homotrimer type I collagen has recently been isolated and reconstituted from fibroblasts of probands with osteogenesis imperfecta (McBride et al., 1992).

The aims of the present study were to investigate the occurrence of our putative type I trimer collagen in breast and colon carcinomas, as well as in some embryonic and fetal tissues, and secondly to acquire additional information on its biochemical properties. We report here the results obtained with analyses performed by high-resolution 2D electrophoresis, enzyme treatments, and peptide mapping. In addition, we have investigated the capability of this collagen to interact

† Supported by MURST (40%), AIRC, and CNR (ACRO 9202249.PF 39). M.A. is the recipient of a fellowship by AIRC (Associazione Italiana per la Ricerca sul Cancro).

* To whom correspondence should be addressed.
‡ Dipartimento di Biologia Cellulare e dello Sviluppo, Università.
§ Centro di Oncobiologia Sperimentale.
∥ Istituto di Istologia ed Embriologia, Università.

0006-2960/93/0432-7421$04.00/0

with laminin, which is also primarily involved in the process of invasion and metastasis of malignant tumors (Hunt, 1989; van den Hooff, 1989). Interestingly, recent data produced by Albini et al. (1992) have indicated the production of a laminin-binding collagen by a neoplastic cell line.

We demonstrate that the previously interpreted type I trimer collagen in IDC can be resolved into three distinct ~100 kDa polypeptides by 2D electrophoresis, namely, an unusual acidic component and two others corresponding to α_1(I) and α_1(III) collagen chains. All three components are involved in the laminin binding. The genetical nature of this collagen remains undetermined, but its peculiar biological properties and occurrence, in both cancer and embryonic–fetal tissues, makes this protein a potential powerful stromal marker of malignancy. We therefore suggest temporarily terming this collagen OF/LB collagen (onco–fetal, laminin-binding collagen).

MATERIALS AND METHODS

Tissue Samples. The sources of collagen used in the present experiments included the following: (i) biopsy fragments of IDC of the breast; (ii) biopsy fragments of colon carcinoma; (iii) hairless fetal calf skin (estimated age: 4 months); (iv) fetal intestine from the same sources as in (iii); (v) human umbilical cord; (vi) human normal dermis, mammary dysplasia, and intestinal polyposis. Tissue samples were kept frozen at –80 °C until used, except for bovine embryo tissues, which were freeze-dried.

Collagen Preparation. Tissues were mechanically homogenized at 4 °C in 10 volumes of Tris-HCl, 0.05 M, pH 7.2, containing antibiotics and protease inhibitor cocktail. The homogenate was stirred overnight in the cold, washed three times with the same solution and once with distilled water, and then transferred to 0.5 M acetic acid overnight. All washings were followed by centrifugation at 18 000 rpm using a JA 20 rotor (Beckman). After tissue swelling, pepsin was added to the suspension to a final concentration of 0.1% pepsin/g of fresh tissue in 0.5 M acetic acid, and suspensions were incubated for 18 h in the cold. For the umbilical cord, a preliminary digestion with 110 units of testicular hyaluronidase/mg of fresh tissue (type IV-S from bovine testis; Sigma, St. Louis, MO) was carried out before the pepsin digestion. After pepsin treatment, material was centrifuged at 18 000 rpm for 1 h and, if necessary, further diluted with 0.5 M acetic acid to a collagen concentration of approx. 0.1 mg/mL (monitored at λ = 230/280 nm). The pH of the supernatant was slowly brought to 7.2 by adding NaOH dropwise under continuous stirring and pH-meter monitoring. For embryonic and tumoral tissues, as neutrality was approached, the solution started to become cloudy; the precipitation was allowed to proceed for at least 2 h in the cold, and the material was recovered by centrifugation at 18 000 rpm for 1 h, resolubilized in 0.5 M acetic acid, and reprecipitated two or three times by the same procedure. The final precipitate was dialyzed against distilled water and lyophilized. This fraction is designated P-1 throughout the text. The supernatant of P-1 was precipitated with 2 M NaCl, and material was recovered as described. This fraction is designated P-2. Final precipitation was achieved by raising the NaCl concentration to 4 M, and this last fraction is designated P-3.

Collagen fractions were assayed by conventional SDS–PAGE (6% acrylamide) with or without reduction with β-mercaptoethanol. Standard collagens were type I from calf skin (Sigma), 97% pure type III from human placenta (Imedex, Lyon, France), type IV (Sigma), and type V from human placenta (Sigma).

Two-Dimensional Polyacrylamide Gel Electrophoresis. First dimension: Nonequilibrium pH gradient gel electrophoresis (NEPHGE) was performed essentially according to the procedure of O'Farrell et al. (1977) using pH 3–10 and pH 5–7 ampholytes purchased from Pharmacia (Uppsala, Sweden) in a 4% acrylamide gel. Before the run, the samples were denatured at 60 °C for 30 min in the presence of 2.5% Nonidet, 4 M urea, and 4% ampholyte mixture with or without 50 mM dithiothreitol. Electrophoresis was carried out in a minigel apparatus (EC Apparatus Corporation, St. Petersburg, FL) for 5 h at 5 V/h.

Second dimension: At the end of 1D electrophoresis, the gel was cut into strips, and each was equilibrated with sample buffer (with or without β-mercaptoethanol) and applied to a 6% SDS–PAGE minigel slab (Bio-Rad, Richmond, CA). Bands were stained with Coomassie Brilliant blue R-250.

Trypsin Digestion. Lyophilized collagen samples (2 mg/mL aliquots) were dissolved in 0.5 M Tris-HCl, pH 7.5, by stirring overnight in the cold. Insoluble material was removed by centrifugation, and aliquots of approx. 100 μg/mL were incubated with 5 μg/mL trypsin (type II; Sigma) for 3 h at 25 °C. Controls were incubated in parallel without trypsin. The reaction was terminated by the addition of 5 mM PMSF, and aliquots of each sample were diluted 1:1 with 2 volumes of electrophoresis buffer containing 1% (by volume) sodium dodecyl sulfate and 1% glycerol. For type III collagen samples 1% (m/v) dithiothreitol was added to the buffer. Prior to the electrophoretic run in a 6% SDS–PAGE minigel system (BioRad), the samples were thermally denatured at 90 °C for 3 min.

Matrix Metalloproteinase (MMP) Digestion. Purified preparations of MMP1, -2, -3 and -9 were kindly donated by Dr. D. E. Woolley (University of South Manchester, U.K.). Collagen substrata (approx. 100 μg/mL) were incubated with 10 μg/mL MMP1, 4 μg/mL MMP9, and 2 μg/mL MMP2 and -3. Enzymes were maximally activated with (4-aminophenyl)mercuric acetate (APMA) as described (Salamonsen et al., 1991).

Cyanogen Bromide Digestion. Lyophilized collagen samples were dissolved in 70% formic acid and digested with CNBr (50 mg/mL in formic acid) overnight at room temperature in the dark under N_2. Reaction was stopped with 10 volumes of distilled water, and the material was dessiccated in a Speedvac apparatus. Digestion products were analyzed by 12.5% SDS–PAGE in a minigel apparatus.

In situ digestion, under the same conditions, was performed on selected bands excised from 2D SDS–PAGE after a brief Coomassie stain. CB fragments from bovine skin, purified by gel filtration on 0.5 M BioGel A, were kindly donated by Prof. R. Tenni (University of Messina, Italy).

Laminin-Binding Assay. Laminin prepared from EHS mouse sarcoma and purified to homogeneity was a kind gift of Dr. M. E. Sobel (NIH, Bethesda, MD). Five milligrams of laminin was coupled to Sepharose CL-4B (Pharmacia) by following the manufacturer's instructions, and the resin was loaded onto PolyPrep columns (Bio-Rad) and equilibrated with Tris-HCl, 0.05 M, pH 8, containing 150 mM NaCl. P-1 collagen fractions were solubilized in equilibration buffer and diluted to an optical density of 0.5–0.8/mL (λ = 230 nm). A total amount of collagen corresponding to OD 2–3 was loaded into the column for each assay. The material was chromatographed at 4 °C, and the unbound material was recovered in 1-mL fractions, which were read at 230 nm. The column was washed with at least 5 volumes of equilibration buffer before elution. The elution buffer was composed of 4, 6, or 8 M urea in 0.05 M Tris-HCl, pH 8, and the eluted material was recovered in 1-mL fractions, which were read at 230 nm. Both unbound and bound material was exhaustively dialyzed against

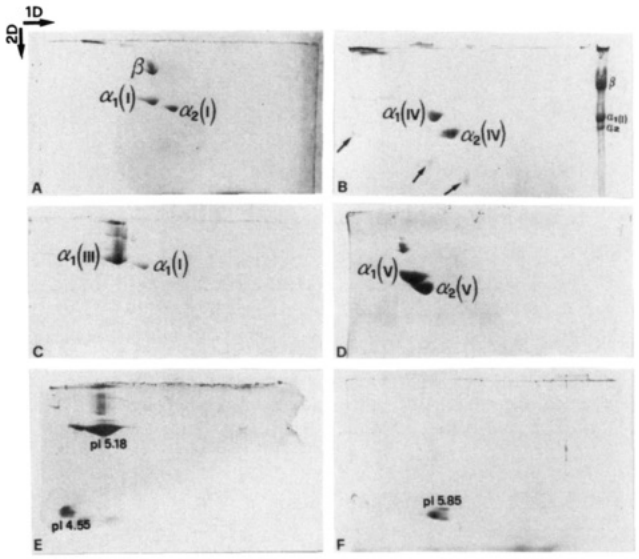

FIGURE 1: 2D PAGE electrophoretograms of collagen types I (A), IV (B), III (C), and V (D) and p*I* molecular markers (E, F). The 1D arrow indicates the NEPHGE separation from acidic to basic pH. The slabs are aligned to permit a comparison between the various chains. All collagen chains tested migrate in the pH range 6–8. α_1(III) is slightly more acidic than α_1(I), while α_2(I) and α_2(IV) appear to be the more basic chains. In gel B, type I collagen was run in the first dimension as molecular weight marker, applying the sample in the single well at the tail of the PAGE slab. The arrows in gel B point to minor spots present in the type IV collagen preparation, and in gel C a small α_1(I) contaminant is detectable. The pH gradient (pH 3–10 and 5–7 ampholytes) was checked with p*I* molecular markers (bovine serum albumin and soybean trypsin inhibitor in gel E; carbonic anhydrase in gel F). Coomassie stain.

distilled water, lyophilized, and stored at –20 °C until used.

Electron Microscopy. Chromatographed collagen fractions were reconstituted in vitro as reported elsewhere (Pucci-Minafra et al., 1986, 1987), and drops of the suspension were applied onto collodion-coated grids and negatively stained with 0.2% potassium phosphotungstate, pH 7.2. A Philips EM 420 electron microscope was used. It was operated at 80 kW with a 150-μm condenser aperture and a 50-μm objective aperture.

RESULTS

P-1 Collagen Preparations Contain an Acidic Component Distinct from α_1(I) Chain. We have previously reported that IDC of human breast contained a collagen fraction precipitating at low ionic strength from neutral solution, which when examined by conventional SDS–PAGE under nonreducing conditions appeared mainly composed of ~100-kDa chains migrating as α_1(I) collagen chains. In the present work we have applied the same protocol to the other fetal and tumoral tissues indicated in Materials and Methods. The corresponding fractions (P-1) represented from 10% to 20% of the total collagen extracted from all tissues analyzed. Calculation was based on the weight of lyophilized material recovered after sequential precipitations as described. By contrast, P-1 collagen was undetectable in adult human nontumoral tissues, such as mammary dysplasia (Luparello, 1987), intestinal polyposis, and human dermis (data not shown).

To test the possible presence of charge microheterogeneity within P-1 collagen fractions, we submitted aliquots from the different sources to a two-dimensional electrophoretic separation, utilizing a NEPHGE/SDS–PAGE procedure. The NEPHGE is not devised to focus the p*I* of molecules, because it is based upon a nonequilibrium pH gradient, but it is widely used to separate proteins with small charge differences from a mixture (O'Farrell, 1977). We have standardized the system with a panel of well-known collagens, namely, types I, III, IV, and V. Figure 1 illustrates the 2D separation of the different collagen chains run in parallel with standard molecular

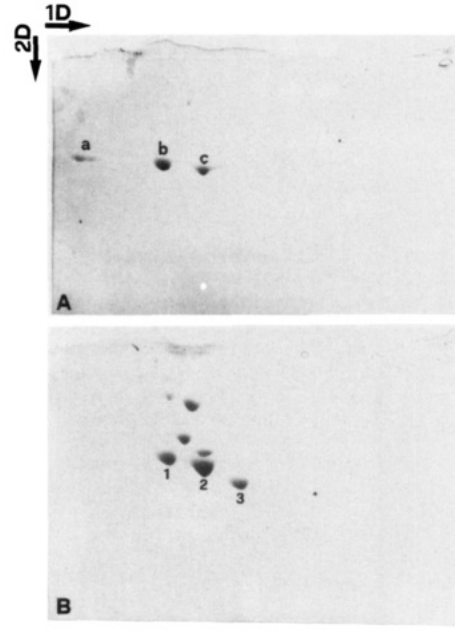

FIGURE 2: 2D electrophoretograms of P-1 (A) and P-2 fractions (B) from bovine embryo skin. In P-1 is present an acidic component (a) absent in P-2, while components b and c correspond to α_1(III) (1 in gel B) and α_1(I) (2 in gel B), respectively. No α_2(I) (3 in gel B) is detectable in P-1 preparations. Coomassie stain.

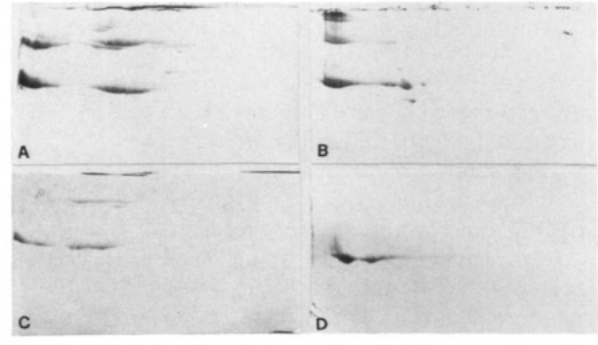

FIGURE 3: 2D electrophoretograms of crude P-1 preparations from bovine fetal intestine (A), human umbilical cord (B), colon carcinoma (C), and breast carcinoma (D). All preparations display a prominent acidic component and poorly resolved components b and c. Coomassie stain.

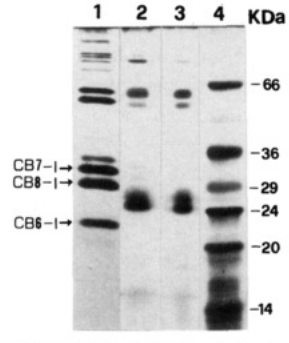

FIGURE 4: 12.5% SDS–PAGE of CNBr-derived peptides from type I collagen (1) and acidic P-1 component from umbilical cord (2) and breast carcinoma (3). Digestion was performed in situ after excision of the acidic bands from 2D PAGE gels, and peptides were revealed with silver stain. The two acidic chains tested produce similar major peptide(s), different from those of type I collagen. Smaller fragments are not detected in this assay because they are probably lost during the procedure or, alternatively, because they are not revealed by the silver stain. (4) Low molecular weight markers (Sigma).

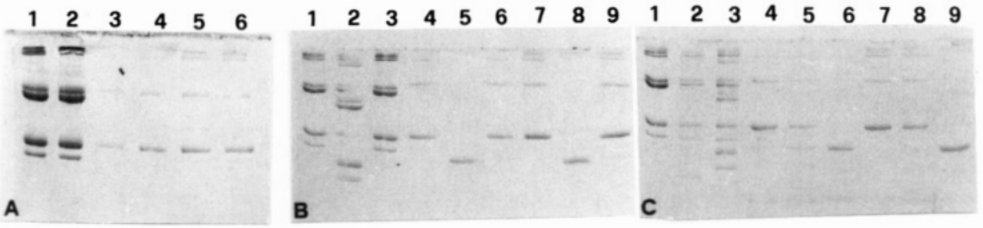

FIGURE 5: (A) 6% SDS–PAGE of type I (1, 2), type III (3, 4), and umbilical cord P-1 collagens (5, 6) incubated in the presence (1, 3, 5) or absence (2, 4, 6) of trypsin. Trypsin is completely ineffective on type I and P-1 collagens, while a modest effect on stain intensity is observed with type III collagen. Before the run, type III collagen was reduced with β-mercaptoethanol. Coomassie stain. (B) 6% SDS–PAGE of type I (1–3), type III (4–6), and umbilical cord P-1 collagens (7–9) incubated without MMP (1, 4, 7) or in the presence of MMP1 (2, 5, 8) or MMP3 (3, 6, 9). MMP1 produces similar cleavage products for all three collagens, while MMP3 is almost ineffective. (C) 6% SDS–PAGE of type I (1–3), type III (4–6), and umbilical cord P-1 collagens (7–9) incubated without MMP (1, 4, 7,) or in the presence of MMP2 (2, 5, 8) or MMP9 (3, 6, 9). A mild effect is observed for MMP2, while MMP9 produces partial cleavage of type I collagen and complete depletion of P-1 and type III collagen chains, which generate a major cleavage product of similar size. Coomassie stain.

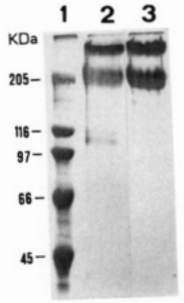

FIGURE 6: 6% SDS–PAGE of (1) high molecular weight markers (Sigma), (2) standard laminin (Sigma), and (3) the laminin preparation used for the present experiments. Coomassie stain.

markers. Figure 2 shows the 2D pattern of a P-1 fraction derived from fetal skin and reprecipitated several times, revealing the presence of three discrete components (indicated as a, b, and c in Figure 2A), having the same M_r of $\simeq$100 kDa but different p*I* values. When compared with the other collagen chains (Figure 1), component a, displaying an apparent p*I* of 4–5, appears unique, while components b and c migrate as α_1(III) and α_1(I) chains, respectively. The acidic component is not recovered either in the supernatant of the P-1 fraction after precipitation with 2 M NaCl (P-2 fraction) which comprises mainly type I and III collagens (Figure 2B) or in the P-3 fraction containing mainly type I and V collagens (not shown). Figure 3 shows 2D electrophoresis panels of P-1 crude fractions obtained from fetal intestine (A), umbilical cord (B), colon carcinoma (C), and breast cancer (D), in which the acidic component is well separated from the poorly resolved components b and c, while α_2 chains are undetectable, indicating a low level of type I collagen contamination (see below).

To check if the acidic component was structurally related to the α_1(I) collagen chain, we performed in situ CNBr digestion, after excision of the acid component from a 2D electrophoretic gel. At least five bands were necessary to visualize the major CNBr-derived fragments with silver stain. Figure 4 shows an electrophoretogram of CB fragments from umbilical cord and breast cancer P-1 preparations run together with α_1(I)-derived fragments. The figure clearly shows that embryonic and tumoral acidic chains are different from α_1(I) chains and produce the same major fragment(s) migrating between CB8(I) and CB6(I) peptides. Smaller fragments are absent, probably due to their loss from the gels during the procedure of the in situ digestion or to their poor reactivity with silver stain.

P-1 Preparations Contain Native Collagen Molecules Sensitive to Some MMPs. Proteolytic assays with trypsin and four different MMPs were performed on P-1 preparations from umbilical cord, in parallel with type I and III collagens. The results of a representative SDS–PAGE analysis are shown in Figure 5. Trypsin digestion (Figure 5A) for 3 h at 25 °C exerted no effect on this collagen, since the stain intensity of γ, β, and α bands remained unchanged with respect to the untreated control. As expected, trypsin was also ineffective on type I collagen, while a modest effect on type III collagen was detectable. The resistance of P-1 collagen to trypsin treatment suggests that this preparation is composed only of native triple-helical molecules.

The results of P-1 collagen treatment with the four MMPs are shown in the electrophoretic analysis reported in Panel B and C of Figure 5. MMP1 apparently cleaves P-1 in a manner similar to its cleavage of type I and III collagens, and the characteristic 3/4 cleavage product is identifiable for the three substrata. No appreciable alteration in protein band intensity was observed when the three collagen samples were incubated with MMP3, while a mild effect was produced by MMP2 on all the substrata, under present conditions. By contrast, MMP9 produced partial degradation of type I collagen and a complete depletion of P-1 and type III collagen chains, which generated a major cleavage product similar in size to the MMP1-derived 3/4 one. The capability of MMP9 to degrade native type I and III collagens has been recently reported (Okada et al., 1992).

P-1 Collagen Shows Affinity for Laminin. The purity of laminin was checked by 6% SDS–PAGE under reducing conditions (Figure 6). A preliminary set of affinity chromatography assays was done with type I, III, and IV native collagens (Figure 7A). Type I and III collagens were not retained by the laminin-coupled resin, while type IV collagen displayed a modest affinity for the ligand. By contrast, P-1 fractions from the different sources displayed a strong affinity to laminin in terms of percentage of bound collagen and of concentration of urea needed to elute the bound fraction. In fact, preliminary assays showed that neither 4 or 6 M urea was sufficient to elute all the material from the column (not shown). The binding assay was reproducibly repeated at least three times for each tissue sample, and Figure 7B-F shows the chromatographic profiles of P-1 collagen from fetal calf skin, breast carcinoma, umbilical cord, colon carcinoma, and fetal intestine. Differences in the unbound/bound fraction ratio among the different samples probably reflect the purity of the preparations used. However, the best recovery was for fetal calf skin (83%; average of five experiments = 69%), while for umbilical cord, breast carcinoma, colon carcinoma, and fetal intestine the bound material accounted for 58, 50, 34, and 28%, respectively (average of three experiments). The P-2 and P-3 fractions collected from the supernatant after P-1 were not retained by the laminin column. The SDS–PAGE assay of bound and unbound P-1 fractions is shown in Figure 8. The bound material migrates as single α_1-sized chains,

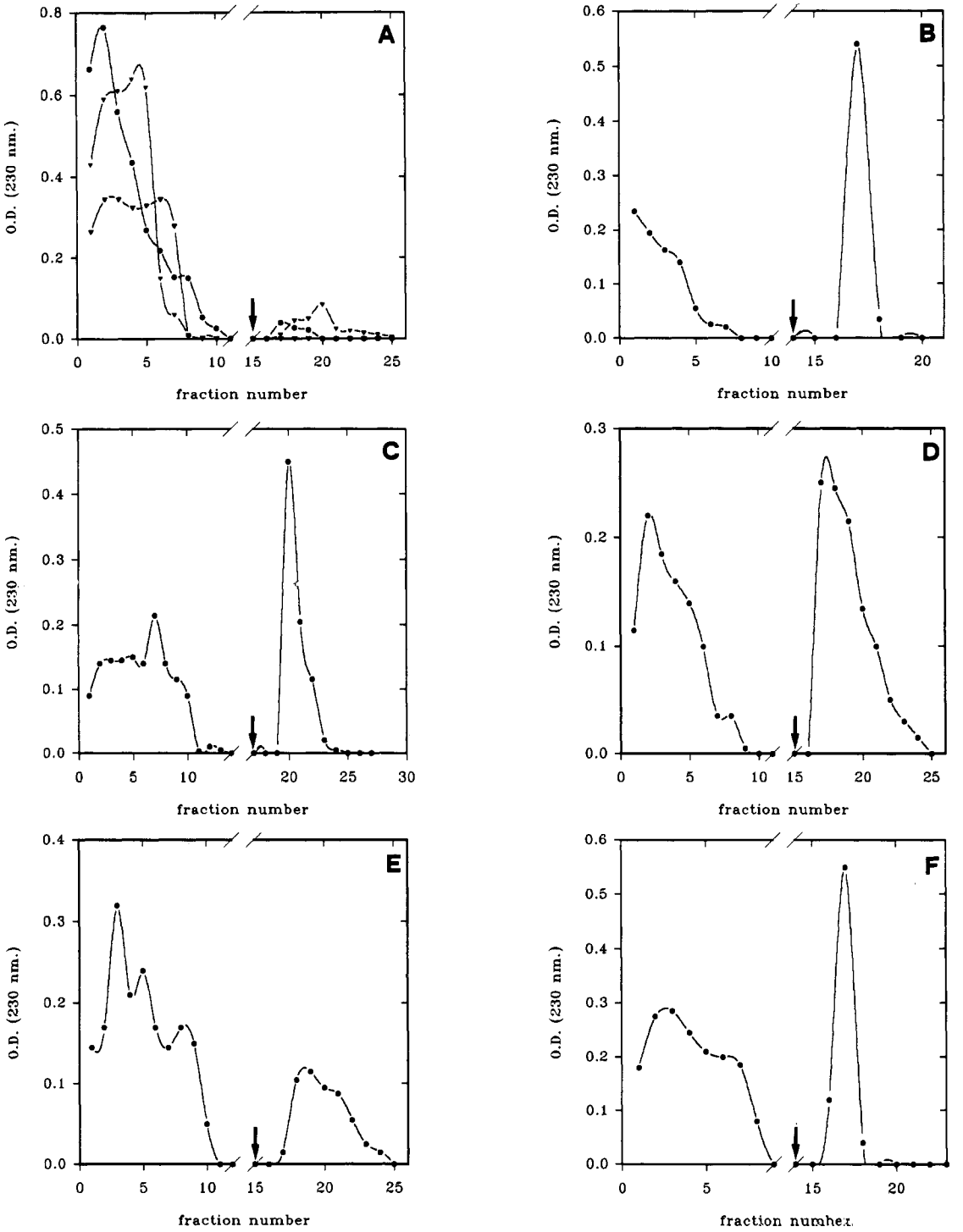

FIGURE 7: Affinity chromatography of collagens on laminin–Sepharose columns. The arrows indicate 8 M urea addition. (A) Type I (●), III (▽), and IV (▼); (B) fetal skin P-1; (C) breast carcinoma P-1; (D) umbilical cord P-1; (E) colon carcinoma P-1; (F) fetal intestine P-1.

both with and without reduction, while the unbound fraction shows a band corresponding to α_2(I) chains: this suggests that the excluded fraction is mainly, if not entirely, composed of type I collagen, as is also supported by the 2D separation (Figure 9A). On the contrary, when submitted to 2D electrophoresis, the bound fraction is resolved into the acidic chain and two other spots with the mobilities of components b and c, respectively (Figure 9B). This result suggests that the interaction with laminin requires the presence of all three chains in native conformation. Furthermore, the material eluted from the column with 8 M urea, i.e., that which underwent denaturation during elution, when resubmitted to affinity chromatography under the same conditions, showed that up to 75% was unretained (not shown).

CNBr Fragments of the Laminin-Bound P-1 Collagen. Lyophilized samples of laminin-bound P-1 fractions were digested with CNBr, and the fragments, separated by 12.5% SDS–PAGE, were compared with type I and type III CNBr-derived peptides. The results reported in Figure 10 show that the laminin-bound collagen produces a couple of peptides comigrating with CB7(I) and CB8(I), a large fragment comigrating with CB5(III), and additional peptides of smaller M_r which appear to be unrelated to type I and III collagens (both human and bovine). The fragment which migrates as CB5(III) was already observed after in situ digestion of the acidic chain.

Fibril Reconstitution. Since the urea concentration required for collagen denaturation starts from 4 M on, we wanted to

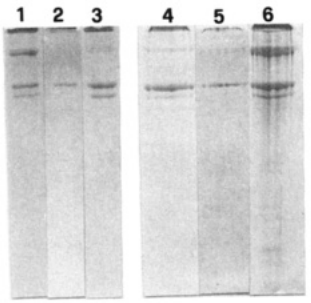

FIGURE 8: 6% SDS–PAGE of unbound (1, 4) and laminin-bound fetal skin P-1 (2, 5). Samples in lanes 4 and 5 were reduced before the run. Lanes 3 and 6 contain standard type I collagen. The material bound to the resin migrates as a single 100-kDa band of similar intensity with and without reduction. The unbound materials reveals a band migrating as α_2(I), due to the relative enrichment of type I collagen in the excluded fraction. Coomassie stain.

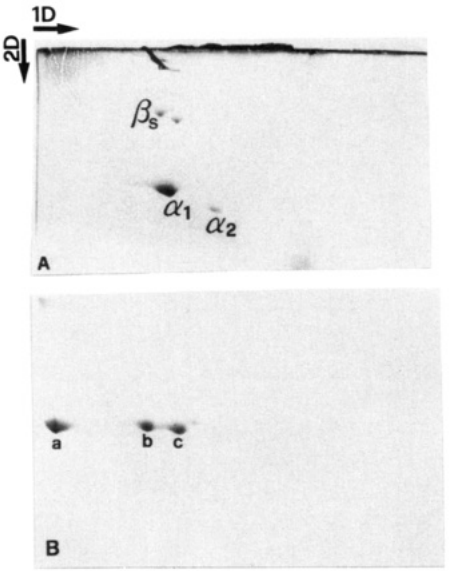

FIGURE 9: 2D PAGE electrophoretogram of the same unbound (A) and laminin-bound collagen (B) as in Figure 8. The unbound fraction appears to be prominently composed of type I collagen (α_1/α_2 ratio > 2) but lacking the acidic chain. The bound material contains all three chains previously indicated as a, b, and c in Figure 2A.

check if the collagen eluted from the laminin column with 4 M urea retained the aggregation properties of native molecules. Both unbound and laminin-bound collagen preparations were submitted to in vitro reconstitution as previously described. As shown in Figure 11, the unbound component produced cross-striated fibrils of small diameter, while the bound collagen fractions formed a thick gel of very thin fibrils with no apparent banding pattern. As expected, poorly organized polymers were formed by the 6 and 8 M urea eluted material (not shown).

DISCUSSION

The present results demonstrate that human breast and colon carcinoma biopsy fragments contain a peculiar form of collagen, not described before, absent in the nontumoral tissues and detectable in almost equal amounts in bovine embryonic tissues and human umbilical cord. This collagen preparatiion (termed P-1 in the text) is equivalent to that previously interpreted as type I trimer (see introduction) because it is composed of identical non-disulfide-bonded α_1-sized chains. In the present report we have shown that when analyzed by 2D electrophoresis, the P-1 fraction was resolved into more than one component of similar size but different charge. The comparison of P-1 collagen with the other collagens tested demonstrated that it contained a unique acidic component,

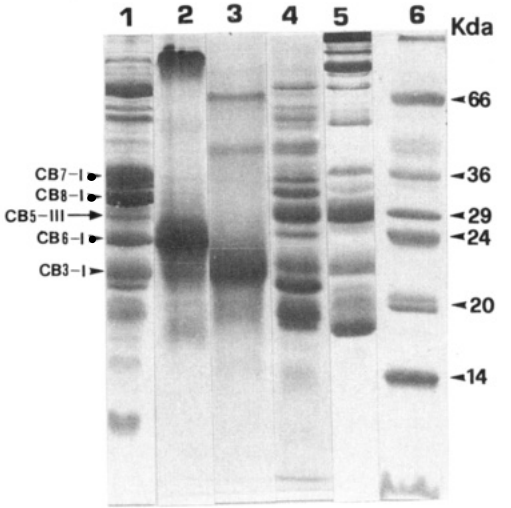

FIGURE 10: 12.5% SDS–PAGE of CNBr-derived fragments from type I (1), embryonic P-1 (4), and type III collagens (5). P-1 collagen shows a couple of fragments similar to CB7(I) and CB8(I), a major fragment corresponding to CB5(III), and additional smaller fragments which appear to be unrelated to both type I and type III collagens. (2) Chromatographically purified CB6(I) peptide; (3) chromatographically purified CB3(I) peptide; (6) low molecular weight markers (Sigma). Coomassie stain.

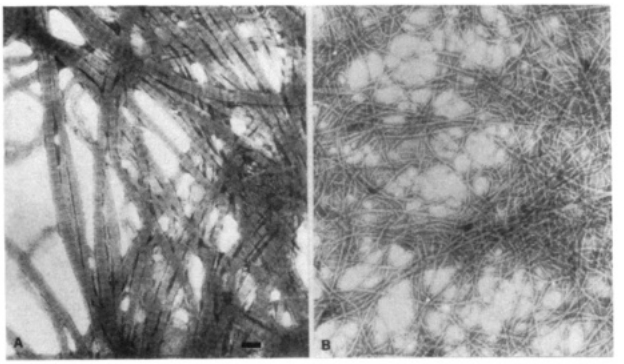

FIGURE 11: Electron micrographs of unbound (A) and laminin-bound P-1 fractions (B) eluted with 4 M urea, reconstituted in vitro, and negatively stained. Under the same conditions, the unbound material produces typical cross-striated fibrils of small diameter, while the fraction isolated by affinity chromatography forms a sticky gel of tiny fibrils in which the cross-striation is undetectable. Bar = 148 nm.

structurally distinct from the α_1(I) collagen chain, and two other components (when adequately resolved) migrating as α_1(III) and α_1(I) collagen chains. The acidic chain is presumably incorporate into stable triple-helical molecules, since the trypsin treatment of P-1 preparations exerted no effect on collagen chain depletion under the same conditions in which a modest effect was observed for intact type III collagen. The partial susceptibility of native type III collagen to trypsin has already been reported by Miller et al. (1976).

A further assay which can provide information on the nature of unknown collagens is the susceptibility to different matrix metalloproteinases. MMPs are a complex family of enzymes, collectively able to degrade extracellular matrix components, but with different substrate specificities. At present the members of this family have been grouped into 4 major subclasses: interstitial collagenases (MMP1 and -8), gelatinases (MMP2 and -9), stromelysins (MMP3 and -10), and matrin (MMP7) (Okada et al., 1992). The susceptibility of the P-1 preparation to MMP1 and resistance to MMP2 and -3 indicate that this collagen belongs to the group of fibrillar collagens; however, it also appears to be cleaved by MMP9 to the same extent as type III collagen, under incubation conditions in which type I collagen is only partially cleaved.

The ability of MMP9 also to attack type I and III collagens has been recently reported by Okada et al. (1992).

One of the most intriguing and interesting properties of the P-1 collagen preparation (at least a large part of it) was the ability to bind strongly to laminin under conditions in which type I, III, and IV collagen, as well as the P-2 and P-3 fractions derived from the supernatant of P-1, did not. The low affinity of laminin for interstitial collagens has long been known (Terranova et al., 1980), while more controversial has been the problem of laminin and type IV collagen interaction, for which direct or nidogen-mediated models have been proposed [references in Engel (1992)]. In our system we have observed very little affinity of type IV collagen (Sigma) for the laminin-coupled resin, while a strong interaction of native P-1 preparations with laminin was testified by the requirement of 8 M urea as efficient eluant. There is also indication that the binding activity is related to the quaternary structure of the collagen, rather than to the primary structure of single chains. This is suggested by the following considerations: (i) After the 2D electrophoretic separation, the laminin-binding collagen was resolved into the three characteristically distinct chains. (ii) When laminin-binding collagen was submitted to CNBr digestion, the overall peptide pattern of this collagen appeared as a combination of α_1(I)- and α_1(III)-derived peptides, plus a few unrelated fragments, confirming that the three distinct components of ~100 kDa are not due to artifacts of the 2D electrophoretic separation. (iii) When the 8 M urea eluted collagen was resubmitted to a second cycle of affinity chromatography, the binding activity was partially lost. A simple explanation of the last observation is that 8 M urea, even when used in the cold during chromatographic elution, is sufficient to denature a large proportion of the collagen applied to the column into single chains, and that the latter lose the ability to rebind to laminin. On the other hand, we have also observed that the material eluted from the column with 4 M urea reconstituted into fibrillar aggregates, even though apparently lacking a clear cross-striation pattern, while the 6 M and 8 M urea eluted material did not. Similarly, it has been shown that the interaction between type I collagen and heparin requires the triple-helical conformation of collagen (Keller et al., 1986).

Concerning the genetic nature of the acidic chain, it can be excluded that it is related to type I collagen, while it appears more related to type III collagen, at least for the major CNBr fragments migrating as CB5(III).

In conclusion, present results indicate that the P-1 preparations, obtained from embryonic and tumoral tissues, contain native triple-helical collagen molecules in which an acidic chain is included. The theoretical possibilities are the following: (i) The P-1 fraction contains a new heterotrimeric collagen type with the chain composition α_1(I)α_1(III)α_1-(acidic), plus a minor quantity of type I and probably type III collagens. (ii) P-1 contains a mixture of collagen types, namely, type I trimer, an unusual type III apparently lacking disulfide bonds, plus a minor acidic component structurally related to type III. Since native type I and type III collagens show no affinity for laminin, while the yield of the material bound to the column is up to 83% of the crude P-1 preparation, the first hypothesis appears more plausible. This hypothesis does not exclude the existence of true type I trimer collagen in our and in other systems, and it also does not necessarily imply that the acidic component by itself is responsible for the laminin interaction; on the other hand, it strongly suggests the existence of a new molecular collagen form, distinct from regular types I and III, which is produced in embryonic and tumoral tissues. We therefore propose temporarily terming this collagen OF/LB collagen (onco–fetal/laminin-binding collagen). Future investigations will be performed to define its molecular identity.

The presence of OF/LB-collagen in embryonic–fetal tissues, its absence in normal adult organs, and its reappearance in cancer strongly suggests the possibility of considering this molecule a stromal marker in malignancy.

ACKNOWLEDGMENT

The authors wish to thank Prof. M. van der Rest (IBCP, Lyon, France) and Dr. D. E. Woolley (University Hospital, South Manchester, U.K.) for constructive criticism of this work and for having offered facilities from their labs. Surgical biopsy fragments were kindly provided by Prof. R. M. Tomasino (Cattedra di Anatomia e Istologia Patologica R., University of Palermo, Italy) and Prof. C. Buccellato (Ospedale Ingrassia, Palermo, Italy).

REFERENCES

Albini, A., Noonan, D. M., Melchiori, A., Fassina, G. F., Percario, M., Gentleman, S., Toffonetti, J., & Chader, G. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2257–2261.
Bornstein, P., & Sage, H. (1989) *Prog. Nucleic Acid Res. Mol. Biol. 37*, 67–106.
Engel, J. (1992) *Biochemistry 31*, 10643–10651.
Hunt, G. (1989) *Exp. Cell Biol. 57*, 165–176.
Keller, K. M., Keller, J. M., & Kuhn, K. (1986) *Biochim. Biophys. Acta 882*, 1–5.
Luparello, C. (1987) *Bull. Mol. Biol. Med. 12*, 49–57.
Luparello, C., & Pucci-Minafra, I. (1986) *Anal. Biochem. 155*, 352–357.
Luparello, C., Schillaci, R., Pucci-Minafra, I., & Minafra, S. (1990) *Eur. J. Cancer 26*, 231–240.
Luparello, C., Sheterline, P., Pucci-Minafra, I., & Minafra, S. (1991) *J. Cell Sci. 100*, 179–185.
McBride, D. J., Jr., Kadler, K., Hojima, Y., & Prockop, D. J. (1992) *Matrix 12*, 256–263.
Miller, E. J., & Gay, S. (1987) in *Methods in Enzymology* (Cunningham, L. W., & Frederiksen, D. W., Eds.) Vol. 144, pp 3–41, Academic Press, Orlando, FL.
Miller, E. J., Finch, J. E., Jr., Chung, E., & Butler, W. T. (1976) *Arch. Biochem. Biophys. 173*, 631–637.
Minafra, S., Pucci-Minafra, I., Tomasino, R. M., & Sciarrino, S. (1984) *Cell Biol. Int. Rep. 8*, 79–85.
O'Farrell, P. Z., Goodman, H. M., & O'Farrell, P. H. (1977) *Cell 12*, 1133–1142.
Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K., & Hayakawa, T. (1992) *J. Biol. Chem. 267*, 21712–21719.
Pucci-Minafra, I., Luparello, C., Sciarrino, S., Tomasino, R. M., & Minafra, S. (1985) *Cell Biol. Int. Rep. 9*, 291–296.
Pucci-Minafra, I., Minafra, S., Tomasino, R. M., Sciarrino, S., & Tinervia, R. (1986) *J. Submicrosc. Cytol. 18*, 795–805.
Pucci-Minafra, I., Luparello, C., Schillaci, R., & Sciarrino, S. (1987) *Int. J. Cancer 39*, 599–603.
Pucci-Minafra, I., Minafra, S., Faccini, A. M., & Alessandro, R. (1989) *J. Submicrosc. Cytol. Pathol. 21*, 475–488.
Salamonsen, L. A., Nagase, H., & Woolley, D. E. (1991) *J. Cell Sci. 100*, 381–385.
Schillaci, R., Luparello, C., & Minafra, S. (1989) *Eur. J. Cell Biol. 48*, 135–141.
Terranova, V. P., Rohrbach, D. H., & Martin, G. R. (1980) *Cell 22*, 719–726.
van den Hooff, A. (1989) *Perspect. Biol. Med. 32*, 401–411.
van der Rest, M., & Garrone, R. (1991) *FASEB J. 5*, 2814–2823.
Vuorio, E., & De Crombrugghe, B. (1990) *Annu. Rev. Biochem. 59*, 837–72.