Role of Collagenous Matrices in the Adhesion and Growth of Cells

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It has long been appreciated that, for many types of cells, attachment to a substrate is required for their replication. Collagen substrates enhance the growth (64, 70, 109, 238) as well as the differentiation (40, 69, 85, 86, 146, 161, 184), of many cells in culture above that observed with other substrates such as plastic and glass. As discussed in this report, many cultured cells including fibroblasts, myoblasts, hepatocytes, chondrocytes, and certain epithelial cells are thought not to bind directly to the collagen substrate or to the plastic surface of culture dishes (31, 73, 74, 76, 77, 89, 93, 113, 164, 220). Instead, extracellular glycoproteins bind the cells to the substrate. One of these attachment proteins, fibronectin, has been extensively studied (99, 151, 166, 227, 228, 244). Fibronectin is produced by fibroblasts (13, 197, 246) and endothelial cells (102, 140) as well as some other cell types. It is also present in high concentrations in blood and serum (152). In culture, serum fibronectin, as well as that produced by the cells, can bind to both the collagen substrates and the tissue culture plastic surface and mediate the attachment of cells (73, 113, 164, 242). Circulating fibronectin participates in a variety of reactions important to wound healing, including the adhesion and spreading of platelets on collagen (10, 14, 75, 204), binding to the fibrin clot (74, 150, 198) and to collagen (37, 46, 94, 103, 119), promoting opsonization reactions by phagocytic cells (18, 94, 201), and promoting fibroblast migration (6, 61). Thus, fibronectin not only functions as an attachment protein, but also may play an important role in repair reactions.

In the case of certain differentiated cells, such as chondrocytes (89) and epithelial cells (159, 220), glycoproteins different from fibronectin are active in attachment. The components of these extracellular matrices of fibroblasts, chondrocytes, and epithelial cells differ with the cell type, and they require separate attachment proteins to provide additional specificity to the interaction of the cell with its matrix. Alterations in cell-substratum interactions are observed in differentiating cells and after spontaneous transformation of cells or exposure to oncogenic agents (99, 228). This review will briefly outline our current knowledge of the types of collagen and their distribution and biosynthesis. Then, the interaction of cells with collagen and other matrix components will be described, with emphasis on cell adhesion, differentiation, and growth.

COLLAGEN

Structure, Biosynthesis, and Degradation

Numerous criteria define a collagen and distinguish it from other proteins (22, 49, 50, 58, 141, 148, 184). Collagens contain a triple-helical segment composed of three chains that have a characteristic composition including 33% glycine, ~10% proline, 10% hydroxyproline, and variable amounts of hydroxylysine. Collagens, but not other proteins, are degraded by bacterial collagenases, which recognize and cleave the sequence gly-X-pro-gly. These, as well as other features such as their x-ray pattern, establish certain structural proteins as collagens and other proteins as containing collagenous domains. At present, two nonstructural proteins having collagenous segments have been identified: Clq, a component of the complement cascade (188), and acetylcholinesterase (192). Detailed molecular models for these molecules have been proposed.

At least five isotypes of collagen molecules have been characterized, and others probably exist (Table I) (22). Although the interstitial collagens, types I, II, and III, are the products of separate genes and have unique amino-acid sequences, in other respects they are quite similar. Each molecule contains a triple-helical domain, roughly 300 × 1.5 nm, composed of three chains, each ~94,500 daltons. Type I collagen, found in skin, bone, and tendon, has two α1(I)-chains and one α2(I)-chain. In addition, collagen molecules containing only α1(I)-chains with the composition [α1(I)]3 have been identified and are usually referred to as type I trimer (142, 157). Type I trimer is found in tissues (105), as products of certain cultured cells (16, 33, 142, 144), and in tumors (149). Type II collagen is found mainly in cartilage and contains three α1(II)-chains (148). Type III collagen, at one time thought to be "fetal" collagen, is composed of three α1(III)-chains and is most prominent in blood vessels, skin, and the parenchyma of internal organs (63). Many investigators consider it to be the reticulin described by histochemical methods, although this has not been proved.
Types IV and V collagens have been described and partially characterized. Type IV collagen is found in basement membranes (22, 110) and probably contains two distinct chains that are larger than the chains of other collagens (222). In addition, type IV collagen has more carbohydrate, 3-hydroxyproline, and hydroxylysine than the other collagens. Type V collagen, which is present in skin, smooth muscle, placenta, and bone (24, 32, 143, 190), contains two types of chains designated αA and αB, or in the new terminology α2(V) and α1(V), respectively. Recent studies indicate that the two chains occur in the same molecule in type V collagen from placenta (15), and molecules containing only αB chains have been isolated from cartilage (190).

The amino-acid sequences of the α1(I)- and α1(II)-chains have been determined, and partial sequences of the α1(III) and α2(I)-chains are known (51). For each chain there is considerable interspecies sequence homology (23).

The steps in the biosynthesis of collagen types I, II, and III have been described (for reviews, see references 50, 141, 183). These collagens are formed from procollagen, a larger, more soluble precursor containing pro-α-chains (Fig. 1). The procollagen chain of type I collagen contains a central collagenous region plus additional peptide sequences of ~13,000 daltons at its amino-terminus and ~36,000 daltons at its carboxy-terminus. Pro-α(I)-, pro-α(II)-, and pro-α(III)-chains contain analogous peptides, which are chemically distinct.

The chains are modified enzymatically after synthesis (Fig. 1) (183). These modifications include the introduction of hydroxyl groups into certain prolyl residues to form 3- and 4-hydroxyproline, and the hydroxylation and glycosylation of some lysyl residues. Most prolyl residues that precede glycyl residues in the molecule are hydroxylated in the 4-position of the ring by an intracellular, probably membrane-bound enzyme, prolyl hydroxylase, which requires for activity ferrous ion, ascorbic acid, α-ketoglutarate, and oxygen (223). Prolyl residues hydroxylated in the 3-position have been found to occur in the sequence 3-hydroxyproline-4-hydroxyproline-glycine (1). Both proline and 4-hydroxyproline stabilize the collagen helix (23, 184), whereas the role of 3-hydroxyproline residues is unknown. Hydroxylation of lysyl residues is carried out by a separate enzyme that has the same cofactor requirements as prolyl hydroxylase (147). In addition, some hydroxylysyl residues are glycosylated by unique sugar transferases to form galactosyl-hydroxylysine and glucosyl-galactosyl-hydroxylysine (58, 183). The function of the sugar residues is not known.

Glycosylation and hydroxylation reactions occur in the pro-α-chains and terminate when the chains assume a helical configuration (183). To form type I procollagen, two pro-α(I)-chains and one pro-α(II)-chain associate, form disulfide bonds at the carboxy-terminus, and coil into a triple helical protein (50). Separate enzymes, procollagen N-protease and procollagen C-protease, remove the amino-terminal and carboxy-terminal peptides, respectively (50, 128, 183). Kinetic studies indicate that the amino-terminal peptides are removed en bloc either inside the cell or at the cell surface, whereas the carboxy-terminal peptides are removed later (50). The liberated amino- and terminal peptides may regulate collagen synthesis by feedback inhibition of translation (163, 239). Because collagen molecules spontaneously aggregate into fibers, it is likely that the carboxy-terminal peptides confer solubility on procollagen and that procollagen C-protease may regulate fiber formation (21, 50). Collagen molecules in fibrils are cross-linked via the condensation of aldehydes formed from certain lysyl and hydroxylysyl residues in the molecule by the enzyme lysyl oxidase (49, 174, 175). Fibrillar type I collagen is readily identified in vivo at the electron microscope level by its characteristic banding pattern (80), whereas the appearance of the other collagens is either

| Collagen type | Composition | Some distinguishing characteristics | Tissue location | Cell types that synthesize various collagen types |
|---------------|-------------|-------------------------------------|----------------|-----------------------------------------------|
| I | α1(I)α2(I) | Low carbohydrate, low hydroxylation of lysine | Skin, bone, tendon, cornea | Fibroblasts, osteoblasts, smooth muscle cells, epithelium |
| II | α1(III)α2(III) | >10 hydroxylsines per chain | Cartilage, cornea, vitreous body | Chondrocytes, neural retina, cells, notochord cells |
| III | α1(III) | Many lysines hydroxylated and glycosylated, sugars other than glucose and galactose, low alanine, high 3-hydroxyproline | Fetal skin, blood vessels, organs | Fibroblasts, myoblasts |
| IV | α1(IV)α2(IV) | Many lysines hydroxylated and glycosylated, sugars other than glucose and galactose, low alanine, high 3-hydroxyproline | Basement membrane | Endothelial and epithelial cells |
| V | α1(V)α2(V) (formerly αA- and αB-chains) | Elevated hydroxylysine, low alanine, contains 3-hydroxyproline | Blood vessels, smooth muscle | Smooth muscle cells, chondrocytes under certain conditions |
less distinct (type II), nonfibrillar (type IV), or unknown (types III and V) (63).

Native collagen molecules and fibrous collagen are resistant to most proteases and the initial step in their breakdown in animals is carried out by a specific collagenase (80). Mammalian collagenase cleaves at a single site approximately one-fourth of the way from the carboxy-terminal end of the molecule (82). The resulting fragments are much less stable than the intact molecule and are presumed to denature and then be degraded by other proteases. There is evidence to suggest that there are collagenases specific for different collagen types. The fibroblast collagenase cleaves collagen types I, II, and III, whereas the neutrophil enzyme cleaves type I but not type III collagen (95). In addition, certain tumor cells produce an enzyme that cleaves type IV collagen (138) but not the others.

Synthesis of Collagen by Cultured Cells

With the exception of lymphocytes, reticulocytes, and other blood-borne cells, most cells in culture synthesize collagen (68, 72, 127). Further, it has been shown that cultured cells can accomplish all the reactions necessary to form and to cross-link collagen fibers (134, 219). However, this is not an efficient process and in culture up to 90% of the procollagen formed accumulates in the medium (131). Much of the procollagen in the medium lacks the amino-terminal peptides but maintains the carboxy-terminal extensions (50). Presumably, the protease that cleaves the carboxy-terminal portions is not sufficiently active in most cells under culture conditions to achieve complete conversion to collagen. Almost complete conversion, however, has been noted with certain mouse fibroblast lines (219).

The amount of collagen produced varies with the cell type. Most fibroblast strains produce collagen as 5–10% of their total protein; whereas other cell types synthesize from 0.001 to 10% of their total protein as collagen (68). Although cells freshly removed from embryonic tendon synthesize collagen as 20–50% of the total protein (36), once in culture, there is a decrease to ~3–5% of the total protein. Relatively minor modifications of the culture medium (Table II), such as the addition of sodium lactate and of ascorbic acid with lower serum levels, allow the cells to maintain a high rate of collagen synthesis (209). Ascorbic acid has long been recognized as essential for the formation of normal connective tissue (26). Although the translation of collagenous polypeptides is not affected in ascorbic acid deficiency, hydroxyproline formation is reduced (173), and the chains do not assume a helical configuration (185). Addition of ascorbic acid has been reported to stimulate proliferation of cells in culture (193), perhaps as a consequence of the enhanced deposition of a collagenous matrix. It should be noted that levels in most media are low and that ascorbic acid has a short half-life (<12 h) in tissue culture (173).

Synthesis of Collagen by Differentiated Cells

Most fibroblasts produce both types I and III collagen simultaneously (62). An even greater variety is observed with smooth muscle cells that synthesize types I, III, IV, and V collagens (143). Epithelial and endothelial cells synthesize type IV collagen, which is consistent with the role of these cells in the formation of the basement membrane (for examples, see references 8, 33, 96, 101, 202).

Freshly isolated chondrocytes synthesize type II collagen, a cartilage-specific protein (224). With time in culture, the cells become more fibroblastic and are found to synthesize type I collagen or both types I and II (16, 231). Late cultures of such cells synthesize types I, II, III, and V, and type I trimer (16, 142). Factors involved in the loss of characteristic chondrocyte activities during culture will be covered in the section Matrix Proteins and Cell Differentiation.

Tissue levels of one or more of the collagens are altered in a variety of diseases (21). In some cases, skin fibroblasts cultured from such patients retain the collagen defect. For example, tissue levels of type III collagen are reduced or absent in patients with the Ehlers-Danlos type IV syndrome (179), and the production of type III collagen by fibroblasts from the skin of these patients is markedly reduced. Fibroblasts from patients with some forms of osteogenesis imperfecta synthesize an unusually high ratio of type III to type I collagen (171). In addition, tumors often synthesize the collagen type characteristic of their tissue of origin. For example, osteosarcomas contain type I collagen (149), whereas chondrosarcomas contain type II collagen (212). In general, the type and amount of collagen synthesized by cells in culture reflect their origins.

RECONSTITUTED COLLAGEN MATRICES

Preparation of Collagen Matrices

Collagen is often employed as a substrate for cultured cells (40, 43–45, 64, 69, 85, 86, 109, 124, 125, 139, 146, 159, 205, 238). Such matrices can be prepared in a variety of forms differing in their properties and components. In many cases, an acid extract of rat tail tendon is dried on a culture dish.

### Table II

| Inhibitors                        | Compounds                  | Mechanism                  | Effect                                      |
|----------------------------------|----------------------------|----------------------------|---------------------------------------------|
| cis-Hydroxyproline (50–200 µg/ml) | Proline analogue           | Inhibits helix formation (4, 183, 194, 225)‡ |
| cis-4-Fluoro-L-proline            |                            |                            |                                             |
| L-Azetidine-2-carboxylic acid     |                            |                            |                                             |
| 3,4-Dehydroproline                |                            |                            |                                             |
| β-Aminopropionitrile (50 µg/ml)   | Inhibits lysyl oxidase     | Inhibits collagen cross-linking (175) |
| a,c'-Dipridyl (0.3 mM)            | Inhibits hydroxylases (iron chelator) | Inhibits prolyl and lysyl hydroxylation (183) |
| D-Penicillamine (1.0 mM)          | Binds to aldehydes        | Inhibits cross-linking (183)     |
| Stimulators                       |                            |                            |                                             |
| Ascorbic acid (50 µg/ml)          | Enzyme cofactor           | Stimulates prolyl and lysyl hydroxylase (173) |
| Sodium lactate (40 mM)            | ?                         | Activates prolyl hydroxylase (223) |

* Amount in parentheses denotes effective concentration for tissue culture studies. 
‡ Numbers in parentheses are references.
Such an extract would consist in large part of type I collagen, but would also have other constituents. Commercial preparations of type I collagen have been used that may contain substantial quantities of impurities including other proteins and even glycogen. Some of the commercial preparations are labeled types I, II, III, IV, and V (Sigma Chemical Co., St. Louis, Mo.). These designations refer to different purification procedures, not to the genetically distinct collagens.

With purified collagens, weighed amounts are dissolved in dilute acetic acid and this solution is placed on the surface of plastic or glass culture dishes. The solvent is evaporated either at room temperature or in a 37°C incubator. 10–50 µg of collagen are sufficient to cover the surface of a 35-mm-diameter dish (121). This substrate is probably not arranged as native-type fibers and may even be denatured (78). A firm and adherent gel can be generated from the acetic acid solution of collagen by exposure to NH₃ vapors (48). Again, the collagen may not be present as native-type fibers.

Heat-reconstituted collagen gels are also used as substrates. In this case, the collagen is dissolved in acetic acid and dialyzed in the cold against 0.15 M NaCl buffered to pH 7.4 with phosphate or Tris. The collagen remains in solution in the cold overnight but forms a firm gel of native fibrils when warmed (44, 81, 121). These gels can be used directly or can be liberated from the surface of the dish to serve as a floating support (45, 205). Higher concentrations of collagen are used to prepare heat-reconstituted substrates (1–4 mg/plate) than for the other coatings and, for this reason, heat-reconstituted gels require higher concentrations of attachment proteins or serum to support cell attachment (121). When nonphysiological levels of phosphate buffer (>0.01 M) are used, calcium and other divalent cations are precipitated from the medium used for cell attachment or culture. Cells attach to this precipitate but often do not spread or divide (121).

**Biological Effects**

Ehrmann and Gey in 1956 (48) were the first to systematically compare growth of many cell strains and tissue explants on collagen substrates with growth on glass. They reported that collagen gels in many cases improved cell growth. Subsequently, it has been observed that collagen substrates alter the morphology (43, 235), migration (181), and adhesion (97, 113, 159) of cells and, in some cases, differentiation (124, 184). In fact, it appears that, for certain cells, growth factors may not be required if the cells are maintained on specific collagens (203) or on the extracellular matrix laid down by the cells (70).

Fibroblasts require a collagen substrate for migration (181) on the plastic filters used in the Boyden-chamber assay. Presumably, the fibroblasts must adhere to the filter in order to move. In addition, collagen α-chains and small collagenous peptides are reported to be chemotactic (180). Thus, collagen and degradation products of collagen may be attractants for fibroblasts in vivo during wound repair, fracture healing, and embryogenesis.

**COLLAGEN AND CELL ADHESION**

Recent studies suggest that specific glycoproteins bind cells to the substratum or to their collagenous matrix. There are a variety of collagen isotypes, and specific binding proteins have been described, while others probably exist. These proteins, of which fibronectin is the best described, are matrix and cell surface constituents. It has been demonstrated that certain cells utilizing these glycoproteins can adhere to specific collagens (Table III). For example, whereas fibroblasts adhere well to all collagens, chondrocytes bind preferentially to type II collagen and epithelial and endothelial cells to type IV collagen. The fibronectin-mediated attachment of fibroblasts to collagen substrates is the most studied. This adhesion is rapid (113), requires divalent cations (113, 115), is temperature dependent (114), and is inhibited by cytochalasin B (168). Fibronectin from one species functions in vitro with cells from other species.

**Fibronectin**

The "cold-insoluble globulin" (CIg) of blood, later identified as a form of fibronectin (198) (Table IV), was originally observed in 1948 in the precipitate that formed when blood was allowed to clot in the cold (150, 228). Although they were first thought to be part of the fibrinogen molecule (41), examination of the amino-acid sequences at the amino-termini of these proteins showed them to be distinct (178). Cold-insoluble globulin is a large molecule (~440,000 daltons) with two similar or identical chains linked by disulfide bonds (152). However, the chains separate slightly on electrophoresis (27) and could be modified forms of the same chain or different gene products.

The protein contains about 6% carbohydrate linked through asparagine residues (25).

The presence of a protein closely related to CIg in connective tissues and on the surface of cells was not discovered until later. In 1973 and 1974, various studies indicated that one of the most abundant glycoproteins on the surface of cells with chains of ~200,000 daltons disappeared after transformation (59, 92, 98, 167, 191, 199, 229, 246). This glycoprotein has been known by a variety of synonyms including cell surface protein (CSP) (246) and "large, external, transformation-sensitive protein" (LETS) (99), but now is referred to as fibronectin (227).

The key role that a serum protein played in mediating the adhesion of trypsinized cells to collagen substrates was reported independently by Haushcka and White (86) and also by Klebe (113). Haushcka and White briefly reported that myoblasts require a serum protein to bind to collagen substrates, but those authors made no further studies. Klebe, while studying the attachment characteristics of established cells, found a serum requirement for fibroblast attachment to collagen substrates (113). Furthermore, he isolated the protein from serum, which he named "collagen-dependent cell attachment protein" (C-CAP). Subsequently, Pearlstein (164) demonstrated that CSP had the ability to promote cell attachment to collagen.

Opsonic proteins that facilitate the phagocytic activities of macrophages are known to be present in serum (3, 18, 94, 200, 241). These proteins, called anti-gelatin factor (241) and α2-SB-glycoprotein (18), were isolated from serum and subsequently shown by independent studies to be identical to serum fibronectin (38, 200). Fibronectin stimulates macrophages to engulf particles coated with gelatin. It also binds DNA (248) and actin (111), presumably also for opsonization.

The fibronectin in blood is not identical in all respects to that in extracellular matrices and on cell surfaces. The blood form is slightly smaller than the cell form (242). Furthermore, after reduction two bands are observed when serum fibronectin is electrophoresed in SDS, whereas a single diffuse band is observed under similar conditions with the cell-associated fibronectin. The serum form is fully soluble at neutral pH, whereas the material extracted from the cells remains in solution best at pH 11. The blood and cellular forms are equally active in mediating cell attachment at levels of 1–5 µg/ml. However, the cellular form is some 50 times more active in
restoring normal morphology to transformed cells (7, 242, 247). The molecular basis for these differences has not been defined but could arise from posttranslational modifications involving glycosylation reactions or proteolysis. Alternatively, the proteins could be genetically distinct molecules.

Current studies indicate that fibronectin is a highly asymmetric molecule in which the monomer has a Stokes radius of 110 Å (245). Physical data suggest that fibronectin contains several globular domains separated by more flexible segments (5, 233). Different fragments of the molecule have been isolated after protease treatment and found to have distinct binding sites as shown schematically in Fig. 2. A fragment of ~40,000 daltons near the amino-terminus contains the collagen-binding site, whereas another fragment of ~160,000 daltons binds to the cell surface (11, 12, 57, 66, 83, 145, 196, 210). The 160,000-dalton fragment does not bind to collagen but can promote cell adhesion to plastic, whereas the collagen-binding fragment (40,000 daltons) lacks cell-attachment activity (83, 84, 196).

One report describes a 50,000-dalton peptide from fibronectin that can both bind to collagen and mediate cell adhesion (66). Thus, although these two regions on the fibronectin molecule are in distinct domains, they could be adjacent. Still another portion of the molecule including the amino-terminal region contains the site cross-linked by transglutaminase (156).

Fibronectin is quite antigenic and antibody to fibronectin has often been used to study its histological distribution (217, 234). Fibronectin is observed in the extracellular matrix around many cells (20, 30, 88, 136, 226, 234) both in regular association with collagen (20, 88, 226) and also as fibronectin filaments lacking any association with collagen (30). Fibronectin is observed on the surface of fibroblasts (197, 226, 246), astrogial cells (229), endothelial cells (102), certain epithelial cells (29), and myoblasts (55). The chondrocytes in cartilage lack the protein but begin to synthesize it when placed in culture (39).

Role of Circulating Fibronectin in Wound Repair

The multiple interactions and biological activities of plasma fibronectin are consistent with it having a role in wound repair. Fibronectin can bind and be cross-linked by transglutaminase (155, 156) to itself (153, 232), fibrin (74, 198, 216), and collagen (11, 12, 37, 46, 47, 66, 67, 78, 83, 84, 94, 103, 104, 106, 119, 120, 145, 164, 165, 196, 210, 242). It is likely that these interactions are essential for the formation of the fibrin clot. As discussed

| Name | Property |
|------|----------|
| Cold-insoluble globulin (CIG) | Coprecipitates with cryofibrinogen |
| Surface fibroblast antigen (SFA), renamed fibronectin | Present on the fibroblast cell surface and in serum and synthesized by fibroblasts |
| Galactoprotein a (Gap a) | Glycoprotein labeled with galactose oxidase–[3H]borohydride that is absent from transformed NIL cells |
| Large external transformation-sensitive protein (LETS) | Cell surface protein lost after in vitro transformation |
| Cell surface protein (CSP) | High molecular weight cell protein on the surface of chick embryo fibroblasts |
| L1 band | Absent from transformed cells |
| Zeta proteins | Absent from transformed chick embryo fibroblasts |
| Band 1 | Absent from transformed BHK cells |
| Collagen-dependent cell attachment protein (c-CAP) | Serum glycoprotein that mediated cell adhesion to collagen |
| Anti-gelatin factor (AGF) | Serum protein required by macrophages to ingest gelatin-coated particles |
| α2-SB-opsonic glycoprotein | Serum protein that promoted phagocytic activity |
| Cell spreading factor (SF) | Serum fraction that promoted cell spreading |

* Numbers in parentheses are references. |
‡ Grotendorst, unpublished observations. 

**TABLE III**

| Adhesion of Cells to Collagen |
|-------------------------------|
| Cell type | Preferred collagen substrate |
|-------------------------|-----------------------------|
| Fibronectin-mediated | I-IV |
| Established cell lines (CHO, 3T3, etc.) (73, 122, 137, 160)* | I-IV |
| Primary fibroblasts (159) | I-IV |
| Rat embryonic periosteum (123) | I-IV |
| Rat hepatocytes (94) | I-IV |
| Osteosarcoma (123) | I-IV |
| Myoblasts (31, 112) | I-IV |
| PMT (highly metastatic pulmonary tumor cell) (158) | I-III |
| Embryonic sheep aortic smooth muscle cells§ (67) | I-III |
| Chondroectin-mediated | II |
| Chick sternal chondrocytes (89) | IV |
| Laminin-mediated | IV |
| Breast epithelial cells (220) | IV |
| Guinea pig epithelial cells (220) | IV |
| EHS (basement membrane producing) tumor cells (220) | IV |
| TERA (transformed epithelial cells) (220) | IV |
| PAM 212 (transformed epithelial cells) (220) | IV |
| PMT (highly metastatic pulmonary tumor cell) (158, 220) | IV |
| Human choroid epithelial cells (220) | IV |
| PYS (pialateral yolk sac cells) (220) | IV |
| Monkey pigmented epithelial cells (220) | IV |
| Sheep aorta vascular endothelial cells (220) | IV |
| Monkey lens epithelial cells (220) | IV |
| Bovine lens epithelial cells (220) | IV |
| Other adhesion mechanisms | IV |
| Platelets (14, 204) | Fibrillar collagen |
| Embryonic sheep smooth muscle cells§ | V |

* Numbers in parentheses are references. 
‡ Grotendorst, unpublished observations.
fibrillar nature of the collagen is also an important factor in
arin has been shown to enhance this binding (104, 106). The
binding is found at physiological temperatures (106) and hep-
collagen occurred more slowly than to denatured collagen and
should be noted that the binding of fibronectin to native
types I-V (37, 46, 47, 103, 119, 123), but not to Ascaris collagen
SPIRING requires fibronectin and cellular metabolism.
Fibronectin will bind to a variety of collagen types including
types I-V (37, 46, 47, 103, 119, 123), but not to Ascaris collagen
(123) or to various synthetic peptides (119). The affinity of
fibronectin for denatured collagen is greater than for the native
protein. For example, Jilek and Hörmann (103) found that
native type I collagen bound 1/100 of the amount of fibronectin
bound by denatured type I collagen at 4°C. Type III collagen
was much more effective under these conditions, binding one-
fourth as well as denatured type I collagen to fibronectin. It
should be noted that the binding of fibronectin to native
collagen occurred more slowly than to denatured collagen and
that equilibrium was not reached at this temperature. Greater
binding is found at physiological temperatures (106) and hep-
arin has been shown to enhance this binding (104, 106). The
fibrillar nature of the collagen is also an important factor in
fibronectin binding, because the binding sites in the collagen
molecules in the internal portions of fibers would be unavail-
able for reaction. Fibrillar type III collagen binds fibronectin
more effectively than type I collagen does (47, 103), perhaps
because type III fibers are thinner (129).
The fibronectin binding site on type I collagen comprises an
unusual sequence of amino acids including the bond cleaved
by mammalian collagenase (37, 120). Cleavage of the α(I)
chain by mammalian collagenase, between residues 775–776,
is sufficient to destroy binding activity (120). A peptide of 35
amino-acid residues, which binds fibronectin and contains the
mammalian collagenase cleavage site (120), has been isolated
from the α(I)-chain. A smaller synthetic peptide including a
part of this region was also active (Fig. 4). The fibronectin-
binding site, like the rest of the helical domain, contains glycine
as every third residue, but lacks proline and hydroxyproline
and is hydrophobic. This region is less stable as a triple helix
than other segments of collagen (91). At physiological tempera-
tures the helix may loosen, allowing fibronectin to bind.
Homologous peptides with binding activity have been isolated
from α2, α(I)II, and α(I)III (37, 117) (Fig. 5). Each sequence
contains the collagenase-sensitive bond, suggesting that the
binding of fibronectin occurs in the same region of each
collagen.
Various other components of the extracellular matrix may
also participate in cell adhesion. Fibronectin binds to proteo-
glycans (172, 243), and heparan sulfate is present in the adhe-
sion sites of cells (35). Fibronectin binds to both hyaluronic
acid and heparan sulfate (104, 213, 215, 243), and the interac-
tion of fibronectin with collagen appears to be stabilized by
both hyaluronic acid and heparan sulfate (195, 243). It is
possible that the relatively low binding of fibronectin to native
collagen is attributable in part to the lack of an additional
factor, such as a proteoglycan that promotes their interaction
(104, 106).
Little is known about the nature of the cell surface com-
ponent that interacts with fibronectin. Certain complex ganglio-
sides with two or three sialic-acid residues have been shown to
bind to fibronectin (117) and inhibit cell adhesion but not alter
the binding of fibronectin to collagen (118). The activity resides
in the carbohydrate portion of the glycolipid. Because ganglio-
sides are cell surface molecules, it is possible that they or other
glycoconjugates with a similar heteropolysaccharide are the
cell surface receptors for fibronectin. A possible model for the
interaction of fibronectin in an attachment site is depicted in
Fig. 6.
The interaction between the cell surface and the fibronectin-
collagen complex is being investigated by isolating and char-
acterizing cell mutants that are defective in adhesion. Poorly
adherent variants of Chinese hamster ovary cells have been

![COLLAGEN BINDING REGION](image)

**FIGURE 2** Schematic model of the fibronectin molecule including the location of specific domains. Peptides containing the collagen-binding site (40K), the transglutaminase cross-linking site (27K), and the cell-binding site (160K) are shown in their probable locations in the molecule (11, 57, 154, 210). Some or all of the sugars are in the 40,000-dalton region (154, 210). The position of the S-S bonds is from Wagner and Hynes (233).

below, fibronectin appears to be involved in some platelet
reactions (10, 14, 204). As already mentioned, fibronectin has
opsonic activity for macrophages (3, 18, 19, 94, 241), which
could be important for debriding the wound. Fibronectin may
also be one of the agents attracting fibroblasts into the wound,
because it is highly chemotactic for fibroblasts (61). In addition,
fibroblasts adhere to fibrin that has covalently bound fibrone-
citin (74). Once in the wound area, fibronectin could help
maintain the fibroblasts by promoting cell-matrix interactions
and stimulating matrix production (53).

**Role of Fibronectin in Cell Attachment to Collagen**

Fibronectin promotes the attachment and subsequent
spreading of many cells (31, 73, 75, 77, 84, 93, 100, 113, 123,
137, 158, 164). In these studies, freshly dissociated cells are
incubated on a plastic or collagen substrate. Most cells will
synthesize their own attachment protein with time but, in short
incubations or when an inhibitor of protein synthesis is present,
the attachment of many trypsinized fibroblasts is stimulated by
serum and by fibronectin (76, 228). Under standard assay
conditions, 50% attachment of Chinese hamster ovary cells is
obtained with ~40 ng/cm² of fibronectin on a substrate con-
taining 100 ng/cm² of collagen. The process is enhanced by
higher levels of fibronectin but is saturable. Three steps in the
process of fibronectin-mediated attachment of cells to collagen
have been described (Fig. 3): (a) fibronectin binds to collagen,
(b) cells bind to fibronectin-collagen complex, and (c) cell
spreading requires fibronectin and cellular metabolism.

Fibronectin will bind to a variety of collagen types including
types I-V (37, 46, 47, 103, 119, 123), but not to Ascaris collagen
(123) or to various synthetic peptides (119). The affinity of
fibronectin for denatured collagen is greater than for the native
protein. For example, Jilek and Hörmann (103) found that
native type I collagen bound 1/100 of the amount of fibronectin
bound by denatured type I collagen at 4°C. Type III collagen
was much more effective under these conditions, binding one-
fourth as well as denatured type I collagen to fibronectin. It
should be noted that the binding of fibronectin to native
collagen occurred more slowly than to denatured collagen and
that equilibrium was not reached at this temperature. Greater
binding is found at physiological temperatures (106) and hep-
arin has been shown to enhance this binding (104, 106). The
fibrillar nature of the collagen is also an important factor in

![FIBRONECTIN](image)

**FIGURE 3** Schematic model of fibronectin-mediated cell adhesion to collagen in vitro. Fibronectin first binds to a specific sequence of amino acids on the collagen chain (113, 120, 165). Then the cells in the presence of Ca²⁺ or Mg²⁺ bind to the fibronectin-collagen complex (115) and spread.
isolated on the basis of their low adhesion to collagen (116). In addition, the mutant cells adhered poorly to glass and tissue culture plastic. Several adhesion-deficient cell strains have been isolated by other selection procedures (169, 182). One mutant has a defect in glycoprotein synthesis but reverts to a normal phenotype in the presence of added N-acetylglucosamine (182). A ricin-resistant fibroblast variant shows defective attachment but synthesizes biologically active fibronectin (169). Analysis of these mutants should help to clarify the interaction of the fibronectin-collagen complex with the cell surface.

Cell-Substrate Adhesion Not Involving Fibronectin

The attachment of baby hamster kidney (BHK) (78, 207) and BALB 3T3 (137) cells to denatured collagen is stimulated by added fibronectin, although some of these cells attach to native collagen without added fibronectin. This has led to the suggestion that different mechanisms are used to attach to the native and denatured protein (137). If this is so, it has not been defined and more likely explanations have not been excluded. For example, cells can utilize for attachment the fibronectin they themselves produce (76). In this case, it would appear that attachment to native collagen was more efficient with the newly synthesized fibronectin. More studies are needed in this area.

Clearly, fibronectin is not involved in the attachment of all cells (Table III). The matrix adjacent to epithelial cells and chondrocyte usually lacks fibronectin (39, 217). These cells abut on extracellular matrices that are quite distinct from fibrous connective tissues. As discussed below, additional glycoproteins, apparently cell specific, attach chondrocytes (89) and epithelial cells (159, 220) to their matrix.

Chondrocyte Attachment — Chondronectin

Cartilage does not contain fibronectin (39, 217), and purified fibronectin does not promote the adherence of freshly isolated chondrocytes, whereas fibronectin-free serum does (89). Chondronectin, the chondrocyte-attachment factor in serum, can be separated from fibronectin by DEAE-cellulose column chromatography. This protein is somewhat more heat-labile than fibronectin (T1/2 = 52°C vs. T1/2 = 57°C) (Table V). As isolated from serum, chondronectin has a molecular weight of 180,000, daltons, and the molecule contains disulfide-linked chains (90). Chondronectin is secreted by cultured chondrocytes and can also be extracted from cartilage; it is presumed to be analogous to collagen in function to fibronectin, but specific for chondrocytes. Interestingly, once chondrocytes attach, fibronectin causes the cells to flatten and appear fibroblastic and, at the same time, the synthesis of cartilage-specific macromolecules is suppressed (170, 237).
TABLE V

| Characteristics of Adhesion Factors |
|-----------------------------------|
| **Fibronectin** | **Chondronectin** | **Laminin** |
| Molecular weight | 450,000 | 180,000 | 800,000 |
| ↓ Reduction | ↓ Reduction | ↓ Reduction | |
| Activity in attachment | 220,000 | 80,000 | 200,000, 400,000 |
| Serum amount | 1-5 µg | 5-50 ng | 1-5 µg |
| Tissue location | 200 µg/ml | Below 20 µg/ml | Below 1 µg/ml |
| Fibrous connective tissue, some basement | Cartilage, vitreous body | Basement membrane |
| membranes | |
| Heparin binding | Yes | Yes | Yes |
| Heat lability | 57°C | 52°C | 49°C |

V). In the presence of laminin (1-5 µg/ml), epithelial cells attach rapidly to type IV collagen. Laminin acts apparently in a manner analogous to that of fibronectin in that it first binds to the substrate, type IV collagen, and then the cells bind to the laminin-type IV collagen complex. Certain cells, such as regenerating liver cells and metastatic cells from the T241 fibrosarcoma (158), attach via fibronectin to a type I collagen substrate and via laminin to a type IV collagen substrate. Because some cells are capable of synthesizing both proteins (53), it can be assumed that these cells utilize both mechanisms for adhesion. In the case of the metastatic cells, the ability to bind to different matrices may be important in their invasive behavior.

**Platelet Adhesion**

The cells in the blood are largely nonadherent. However, in culture, monocytes and neutrophils adhere to all collagen types and to bacteriological plastic (122). It has been suggested that fibronectin is the collagen receptor on the platelet cell surface (14). Platelets contain fibronectin (14, 152, 176), which is released during their aggregation by collagen or by thrombin (249); however, only small amounts are found on the cell surface (65). Antibody against fibronectin, however, only blocks adhesion by 20% and has no effect on aggregation (204). Furthermore, whereas fibronectin binds best to denatured collagen (37, 46, 47, 103, 119), platelets are aggregated by native and not by denatured collagen (60, 240). These observations suggest that fibronectin does not participate in the initial step in the platelet-collagen interaction. Once platelets adhere to collagen, exogenous fibronectin enhances their flattening (75, 100). Recent evidence of a role for fibronectin in platelet aggregation is suggested by studies on a patient with the Ehlers-Danlos syndrome with poor wound healing and abnormal platelet aggregation (10). This patient was found to have normal levels of fibronectin, using an immunological assay. However, only a slight aggregation of the patient's platelets occurred in plasma while the addition of fibronectin from a control subject corrected the defect. These data suggested that the fibronectin from the patient was not functional in platelet aggregation. Clearly, fibronectin is present in platelets and involved in platelet function, but its role as the collagen receptor is unlikely.

**COLLAGEN AND CELL GROWTH**

It is well established that normal cells must attach to a substrate in culture to survive and divide (211). Such cells deposit material on the surface of the dish as they adhere (34, 35, 133, 208). Under appropriate conditions, collagenous protein is identified in the substrate attachment matrix (34, 208). As expected, little collagenous protein is present when ascorbic acid levels in the medium are low (30), because ascorbic acid is necessary for maximal synthesis. Similarly, little collagen is present when transformed cells are used, because they make less collagen than normal cells (2, 71, 107, 135) or fail to convert procollagen to collagen (9). It is likely that the plastic or glass surface is adequate without collagen. Although it is not clear how glass and plastic surfaces interact with the cell membrane, more recent studies suggest that tissue culture plastic adsorbs fibronectin and other glycoproteins that the cells use for attachment (31, 73, 76, 77, 93). In this case, the plastic and glass would serve as a surrogate for the natural collagenous matrix.

Collagenous matrices, however, do alter the growth of normal and transformed cells in culture. Cells such as hepatocytes (205), corneal endothelial cells (69), breast epithelial cells (238), and epidermal cells (159) are maintained in a viable state longer on a collagenous substrate than on plastic. Similarly, a collagen substrate was found to prolong the lifespan of cultured chick fibroblasts (64). Growth-promoting effects have been noted in culture with ascorbic acid (193) and attributed to the ability of ascorbic acid to increase the production of a collagenous matrix (173). Dexamethasone (56) as well as butyrate (87) induce normalization of the morphology in transformed cells along with increasing the deposition of collagen (56) and fibronectin (56, 87) by the cell. Possibly the reduced adhesive-ness of transformed cells and their failure to assume a normal parallel alignment could be attributed to the reduced production of matrix proteins.

Proline analogues that prevent the deposition of collagen have been used to determine the necessity for collagen synthesis during cell culture. Fibroblasts cultured in the presence of cis-hydroxyproline begin to round up within a few hours and eventually detach (108). However, cis-hydroxyproline has no effect on growth (139) when cells are plated on a collagen substrate (as low as 0.1 µg/cm²). This indicates that the cis-hydroxyproline is not toxic to the cells but prevents the cells from producing a collagenous matrix required for growth. Using this analogue, it has been possible to determine the requirement that certain cells have for specific collagens. Breast epithelial cells will not grow on type I collagen in the presence of cis-hydroxyproline but will grow on type IV collagen (238). Thus, these cells specifically require a type IV collagen substrate for growth.

Cis-hydroxyproline inhibits the growth of chick embryo tendon fibroblasts in primary culture (108) and a variety of other cells, but does not affect the growth of several trans-
formed cell lines including KB cells, neuroblastoma cells, and other cells listed in Fig. 7. These data (Fig. 7) suggest that the tumorigenic cells that make only small amounts of collagen are not dependent on collagen for growth.

MATRIX PROTEINS AND CELL DIFFERENTIATION

Possible roles for collagen in differentiation have been assessed by growing cells on collagen substrates, by treating cells or tissues with bacterial collagenase, and by treating cells with drugs that interfere with collagen synthesis (Table VI).

Purified collagen substrates and collagenous matrices have been shown to maintain differentiated functions as well as to induce differentiation in cultured cells. One of the best-documented roles for collagen in differentiation is in the fusion of myoblasts into multinucleated myoblasts (85, 124). Myoblasts are unable to form myotubes when plated in the absence of fibroblasts (125). However, in the presence of conditioned medium (124) or on collagen-coated dishes (85), they form myotubes. Both native and denatured collagen support differentiation, but only when the collagen is used as a substrate and not when it is added to the medium (86). All mammalian collagens tested are equally active in promoting muscle differentiation (112), and it was found that the α1(I)-CB7 peptide, the fibronectin-binding peptide (37, 119), contains the active region (86). A serum factor was shown to be necessary for the attachment of myoblasts to collagen (86), and this was subsequently shown to be fibronectin (31). It is interesting to note that as myoblasts fuse they lose their cell surface fibronectin (28, 55), and the addition of exogenous fibronectin delays fusion (177). Thus, myoblasts appear to require fibronectin for attachment, whereas loss of fibronectin from the cell surface is necessary for differentiation.

The differentiation of a variety of other cell types is enhanced by collagen, including somite chondrogenesis (126, 130), the induction of matrix formation by corneal epithelium (146), and the adhesion and development of some epidermal cells (159). Somite chondrogenesis is improved by type I procollagen and collagen, and type II collagen is even more effective (126, 130). It is possible that the type II collagen synthesized by the notochord induces chondrogenesis in somites in vivo. The production of the epithelial stroma of the cornea is enhanced by all collagens (40). Epidermal cells in culture often require feeder layers of mesenchymal cells (189) or the presence of collagen gels for growth (109). Type IV (basement membrane) collagen has been found to promote the attachment and the subsequent differentiation of epidermal cells into squamous epithelium (159).

One of the most impressive activities of a collagenous matrix is the induction of bone by devitalized and decalcified bone powder implanted in syngeneic hosts (186). This material induces an outgrowth of mesenchyme cells that differentiate into chondrocytes. Subsequently, blood vessels enter the area, and the cartilage is replaced by osteoblasts. Later, osteoblasts and marrow spaces containing hematopoietic cells form (186). The cells at each stage synthesize unique matrix proteins (187), and it is the matrix produced by the osteoblasts rather than the implanted bone powder that mineralizes. In culture, the bone powder also induces chondrocytes to develop from cells in minced muscle tissue (160). It is not clear how the bone powder, which is mostly collagen, acts. However, it has been suggested that the collagenous matrix contains a firmly attached "inducer" molecule (160).

Further evidence that collagen participates in differentiation has been obtained by use of bacterial collagenase or drugs that block collagen biosynthesis. Treatment with collagenase prevents the development of feathers (218), lung (236), and salivary epithelium (79) as well as the induction of notochord cartilage by spinal cord (162). It is possible that noncollagenous macromolecules are also involved, because the collagenases used may have contained other enzymes including proteases. Further, proteoglycans have been implicated as necessary for salivary epithelial morphogenesis (17). Several drugs that inhibit collagen formation, including proline analogues, have been shown to prevent lung morphogenesis (4, 214), salivary gland formation (214), and ameloblast differentiation (194).

Collagen is not the only matrix protein known to influence cell differentiation. For example, the phenotype of chondrocytes is altered by fibronectin. Although cartilage does not contain fibronectin, freshly isolated chondrocytes, when cultured at low cell density in the presence of serum, become flattened, synthesize fibronectin, and retain it on their cell surface (39). These effects are observed in cells grown on plastic substrates but, as expected, occur more rapidly in cells grown on collagen substrates (170). As cell density increases, chondrocytes become less flattened, synthesize increased amounts of proteoglycan, and no longer retain fibronectin on their cell surfaces. Chondrocytes grown in medium with serum depleted of fibronectin are less flattened, produce more proteoglycan, and synthesize and retain less fibronectin than cells grown with fibronectin (170, 237). The "dedifferentiation" of chondrocytes observed in long-term culture (206) could be attributed in part to the alterations in synthetic activities resulting from fibronectin-dependent changes in cell shape.
proteoglycans, it is likely that unique and complex matrices are formed that maintain the cells within the tissue and direct their growth and differentiation. Some examples of the affinity of cells for the different collagen isotypes are known, including chondrocytes that prefer type II collagen, and epidermal cells for the different collagen isotypes are known, including keratinocytes that prefer type IV collagen.

**CONCLUSIONS**

At present there are five well-identified isotypes of collagen that vary in composition, fiber structure, and tissue distribution, suggesting that they play more than just a structural role. The collagen isotypes are known to have somewhat different compositions depending on the type or age of the tissue. Because collagens can interact with various attachment proteins and proteoglycans, it is likely that unique and complex matrices are formed that maintain the cells within the tissue and direct their growth and differentiation. Some examples of the affinity of cells for the different collagen isotypes are known, including chondrocytes that prefer type II collagen, and epidermal cells and breast epithelial cells that prefer type IV collagen.

Although we regard most cells as permanent residents of the tissues to which they belong because of their attachments to other cells and to the extracellular matrix, a variety of evidence suggests that tumorigenic and particularly metastatic cells behave differently. Many of the properties of these cells relate to their reduced adhesion to a matrix and reduced synthesis of fibronectin and collagen. Many tumorigenic cells synthesize less collagen and probably less fibronectin, but synthesize larger amounts of proteases including collagenases. As a result, the cells are less flattened in culture and less dependent on an extracellular matrix for growth. Such factors would also increase their invasiveness.

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**TABLE VI**

| System | Differentiated product | Evidence for matrix protein requirement |
|--------|------------------------|-----------------------------------------|
| Effect of pure collagen substrates | Multinucleated myotube cells | Requires (a) conditioned medium or (b) collagen substrate and a serum component |
| Chick embryonic myoblast (124)* | Multilayered squamous epithelium | Requires (c) type IV collagen substrate |
| Corneal epithelium (40) | Cartilage formation | Requires collagen substrate, type II collagen most effective |
| Chick embryonic somites (126, 130) | Production of stroma | Requires (b) lens as a substratum or (b) collagen substrate |
| Epidermal cells (159) | Cartilage formation | Requires (a) feeder layer of cells, (b) crude collagen substrate, or (a) demineralized bone powders |
| Effect of collagenous matrices | Bone formation | Requires extracellular matrix of retinal pigmented epithelium |
| Subcutaneous implant of decalcified bone (186) | Cartilage and bone | Requires (a) Demineralized bone powders or (b) freeze-thawed bone |
| Muscle mesenchymal cells (160) | Cartilage formation | Requires extracellular matrix of retinal pigmented epithelium |
| Neural crest mesenchymal cells (161) | Cartilage formation | Requires extracellular matrix of retinal pigmented epithelium |
| Effect of enzymes or drugs | Feather morphogenesis | Blocked by collagenase but not trypsin |
| Chick embryonic skin tracts (218) | Branched salivary glands | Blocked by collagenase and by L-azetidine-2-carboxylic acid and α,α'-dipyridyl |
| Embryonic salivary rudiment epithelium (79, 214) | Branched epithelium and maintenance of shape of ureteric bud | Blocked by exposure to collagenase but can be restored by adding back lung or kidney mesoderm |
| Chick embryonic lung epithelium, chick or mouse embryonic ureteric bud (236) | Lung morphogenesis or ameloblast differentiation | Blocked by proline analogue, L-azetidine-2-carboxylic acid |
| Embryonic lung or tooth mesenchyme (4, 214) | | |
| Effect of fibronectin | Cartilage formation | Inhibited by fibronectin, restored by its removal |

* Numbers in parentheses are references.

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