Proc. Natl. Acad. Sci. USA
Vol. 78, No. 5, pp. 3218–3221, May 1981
Medical Sciences

Deposition of plasma fibronectin in tissues

(extracellular matrix/basement membranes/plasma proteins)

ESTHER OH, MICHAEL PIERSCHBACHER, AND ERKKI RUOSLAHTI*

La Jolla Cancer Research Foundation, 2945 Science Park Road, La Jolla, California 92037

Communicated by Ernest Beutler, February 9, 1981

ABSTRACT **Tissue distribution of human fibronectin injected intravenously into mice was studied by using immunofluorescence with species-specific antisera to human and mouse fibronectins. Human fibronectin was detected in the tissues of mice injected with human fibronectin. The distribution of the injected fibronectin was indistinguishable from that of mouse fibronectin. The staining for human fibronectin in the livers of injected mice was unaffected by perfusion of the livers prior to the preparation of tissue sections, and human IgG injected in mice as a control was not detectable in tissues by immunofluorescence. Mice were also injected with fragments of fibronectin with molecular weights close to 200,000. These fragments lack parts of the NH_2 and COOH termini of the fibronectin polypeptide, including the part with the interchain disulfide bond(s), but retain the ability to bind to collagen and heparin and the ability to mediate cell attachment. They showed essentially no tissue incorporation, suggesting that the integrity of the fibronectin molecule is important for the accumulation of fibronectin in tissues. The incorporation of injected fibronectin into tissues demonstrated here suggests that circulating fibronectin contributes to the extracellular matrix of tissues.**

Fibronectin is a high molecular weight glycoprotein present at cell surfaces, in connective tissue, and in blood and other body fluids (for recent reviews, see refs. 1 and 2). There is compelling *in vitro* evidence that cell surface fibronectin mediates the attachment of cells (3–6), and it is generally assumed that tissue fibronectin serves a similar function. Fibronectin at the cell surface and in tissues is insoluble, and its relationship to the soluble forms of fibronectin such as in plasma is not clear. The molecular properties and immunological reactivities of the plasma and cell-associated fibronectins are similar (7–9). The differences that are found in the mobilities of fibronectin from plasma and from some other sources on sodium dodecyl sulfate/polyacrylamide gel electrophoresis seem to be mainly, if not exclusively, due to differences in the extent of glycosylation of the protein (10, 11). Both the plasma and cellular forms of fibronectin mediate cell attachment (3, 4) and bind to collagen (12), glycosaminoglycans (13, 14), and actin (15), but quantitative functional differences between the plasma and cell surface forms have been reported (16).

The function of plasma fibronectin is not known. It has been suggested that it serves as an opsonin, facilitating the removal, by phagocytic cells, of tissue debris containing collagen, fibrin, and other insoluble macromolecules with which fibronectin interacts (17, 18). In this study we show that human plasma fibronectin injected into mice is deposited in tissues where its distribution is indistinguishable from the mouse's tissue fibronectin, suggesting that one of the functions of plasma fibronectin is to provide a reservoir for tissue fibronectin.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Isolation of Fibronectin and its 200,000-Dalton Fragments. Fibronectin was isolated from normal plasma by gelatin-Sepharose chromatography (12). Fibronectin fragments were isolated from a commercial cryoprecipitate of human plasma (a gift from Alpha Therapeutic, Los Angeles). Fibronectin and its gelatin-binding fragments present in the cryoprecipitate were isolated by chromatography on gelatin-Sepharose. The eluted material was dialyzed against water. Material insoluble in water was removed by centrifugation, the soluble part was fractionated further on heparin-Sepharose (Pharmacia), and the bound fraction was collected. The 205,000- and 190,000-dalton fragments (called "200-kDal" fragments here) present in this fraction have lost the region of the molecule that contains the interchain disulfide bond(s) but have retained the ability to interact with collagen and heparin and to mediate cell attachment. The detailed characterization of such fragments will be published elsewhere. Fresh human plasma cryoprecipitate was obtained from a local blood bank.

Proteins were subjected to gel electrophoresis on 5% polyacrylamide slab gels in the presence of sodium dodecyl sulfate with and without reduction by 1% 2-mercaptoethanol.

Injection of Fibronectin and Analysis of Tissues. Mice (C3HeB/FeJ, Jackson Laboratory, Bar Harbor, ME) were injected intravenously with 1 mg of purified human fibronectin, an amount of cryoprecipitate containing 1 mg of fibronectin, 200-kDal fragments, or human IgG (Miles). Fibronectin was desalted in a column of Sephadex G-25 (Pharmacia) equilibrated with 0.9% NaCl to remove urea. The other proteins were lyophilized from water and dissolved in 0.9% NaCl. Cryoprecipitate was solubilized by warming to 37°C. The injections were given in the tail vein on 3 successive days and the mice were bled and sacrificed on the fourth day. The plasma samples were assayed for human fibronectin by radioimmunoassay specific for human fibronectin (19). In some cases, the livers from injected mice were perfused with Eagle's minimal essential medium (aerated with 95% O_2/5% CO_2) at 5 ml/min for 20 min at 37°C prior to immunofluorescent staining.

Tissues for immunofluorescence studies were snap-frozen in liquid nitrogen. Cryostat sections (5-μm) were fixed in acetone at room temperature for 10 min. Fibronectin in the tissues was visualized by indirect immunofluorescence. Fluorescein-conjugated goat anti-rabbit IgG was obtained from Cappel Laboratories (Cochranville, PA). Rabbit anti-mouse IgG and anti-human IgG were from Miles. Rabbit antiserum against human fibronectin was made species-specific by absorption with mouse fibronectin-Sepharose, and antibodies to human fibronectin were isolated from the absorbed serum by adsorption to and

Abbreviation: kDal, kilodalton.

* To whom reprint requests should be addressed.

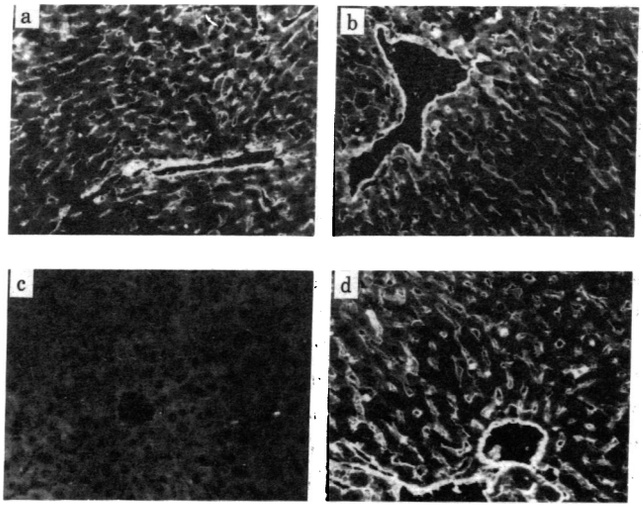

FIG. 1. Immunofluorescent staining for fibronectin in livers from mice injected intravenously with human plasma fibronectin (*a* and *b*) and mice not injected (*c* and *d*). (*a* and *c*) Sections stained with species-specific anti-human fibronectin; (*b* and *d*) sections stained with species-specific anti-mouse fibronectin.

elution from human fibronectin coupled to Sepharose (19, 20). Antibodies specific for mouse fibronectin were prepared similarly by absorption of rabbit anti-mouse fibronectin with Sepharose-coupled human fibronectin followed by isolation of antibodies on mouse fibronectin coupled to Sepharose. Monoclonal antibodies to human fibronectin were obtained by the hybridoma technique (21) using human amniotic fluid fibronectin as immunogen in mice.

RESULTS

Incorporation of Human Fibronectin in Mouse Tissues. The mice injected with human fibronectin had an average blood level of human fibronectin of 77 μg/ml at the time of sacrifice as determined by radioimmunoassay. Fibronectin was localized in tissue sections from these and normal mice by indirect immunofluorescent staining using species-specific antisera. Anti-human fibronectin gave staining in the sinusoidal areas of livers from mice injected with human fibronectin (Fig. 1*a*) but not in the livers of uninjected mice (Fig. 1*c*). The staining pattern was identical to that obtained with anti-mouse fibronectin which stained both types of livers (Fig. 1 *b* and *d*). In the kidneys of injected mice, staining for human fibronectin was present in the glomeruli and around the tubuli (Fig. 2). Again, the staining pattern was similar to that of mouse fibronectin and no staining was seen in the kidneys of uninjected mice. Heart muscle also was found to contain human fibronectin in the injected mice (Fig. 3). Optimal staining in the tissues of injected mice was obtained with the anti-human fibronectin reagent diluted 1:30; the same reagent gave no staining in the tissues of uninjected mice even when used at dilution of 1:10. Five mice were injected with purified human fibronectin and in each case the tissues could be stained with anti-human fibronectin.

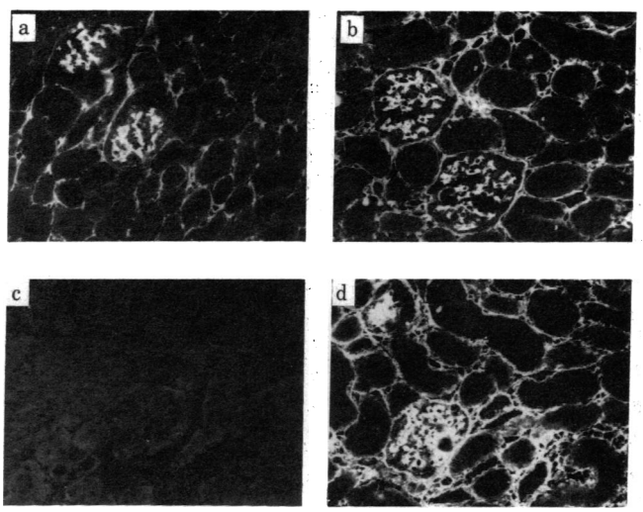

FIG. 2. Immunofluorescent staining for fibronectin in kidneys from mice injected with human plasma fibronectin (*a* and *b*) and from uninjected mice (*c* and *d*) stained with anti-human (*a* and *c*) and anti-mouse (*b* and *d*) fibronectins.

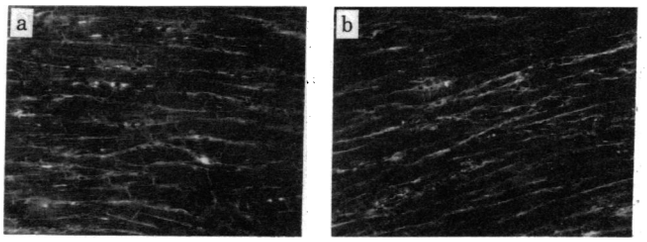

FIG. 3. Immunofluorescent staining for fibronectin in heart tissue of a mouse injected with human plasma fibronectin, stained with anti-human fibronectin (*a*) and anti-mouse fibronectin (*b*).

The specificity of the detection of human fibronectin in the tissues from the injected mice was confirmed with monoclonal antibodies specific for human fibronectin. These antibodies stained tissues from the injected mice but not tissues from normal mice (Fig. 4). Each monoclonal antibody alone gave a weak staining, but a mixture of four antibodies known to react with different antigenic determinants in the fibronectin molecule gave strong staining as shown in Fig. 4.

To exclude the possibility that the purification procedure we used would have altered the fibronectin so that it would become incorporated in tissues, we also injected two mice with fresh plasma cryoprecipitate. A pattern of tissue staining similar to that seen with purified fibronectin was obtained (result not shown).

Other control experiments showed that the human fibronectin staining in the livers was not affected by perfusion of the liver prior to sectioning (Fig. 5*a*), and IgG was not detected by immunofluorescent staining with rabbit anti-human IgG in liver sections from mice that had been injected with human IgG (Fig. 5*b*).

Dependence of Tissue Incorporation on the Integrity of the Fibronectin Molecule. Commercial plasma cryoprecipitate contained intact fibronectin and an array of fragments of fibronectin that were coisolated with fibronectin on gelatin-Sepharose. The fragments were soluble in water but intact fibronectin was not. Part of the gelatin-bound water-soluble material bound

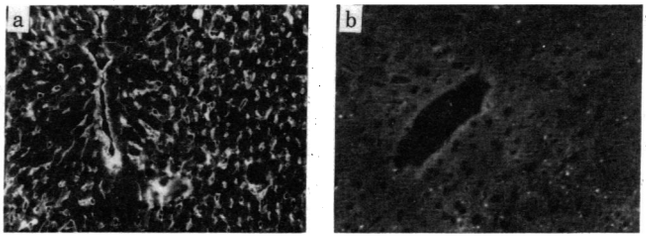

FIG. 4. Immunofluorescent staining with monoclonal antibodies to human fibronectin in livers of a mouse injected with human plasma fibronectin (*a*) and an uninjected mouse (*b*).

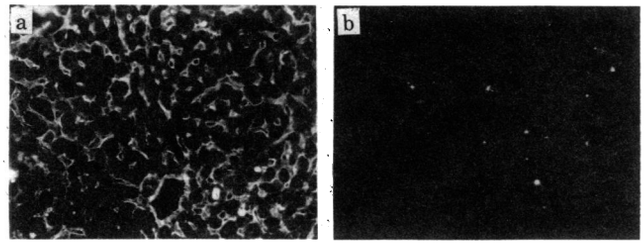

FIG. 5. (*a*) Immunofluorescent staining of the liver of a mouse injected with human plasma fibronectin. The liver was perfused prior to sectioning and staining. (*b*) Lack of immunofluorescent staining for human IgG in tissues of a mouse injected with human IgG.

to heparin-Sepharose. The heparin-bound material contained two major polypeptides with sizes close to 205,000 and 190,000 daltons (Fig. 6). These fragments (200-kDal fragments) migrated with similar mobilities when unreduced and reduced; intact fibronectin migrates with the mobility of a dimer when not reduced. In immunodiffusion against rabbit anti-fibronectin, the 200-kDal fragments reacted to form a precipitate that fused completely with that of intact fibronectin (not shown). In the fibronectin radioimmunoassay, they were slightly more active than intact fibronectin (Fig. 7).

Mice injected with 200-kDal fragments had an average plasma level of 74 μg/ml at the time of sacrifice. When stained with anti-human fibronectin, tissues from such mice showed some staining in the blood vessels and the kidney glomeruli (Fig. 8). However, unlike in the mice injected with intact fibronectin, the tubular areas showed no staining, and only weak and irregular staining was seen in the liver sinusoidal areas.

DISCUSSION

Our results reveal an accumulation of plasma fibronectin in tissues. The distribution of the injected human fibronectin in

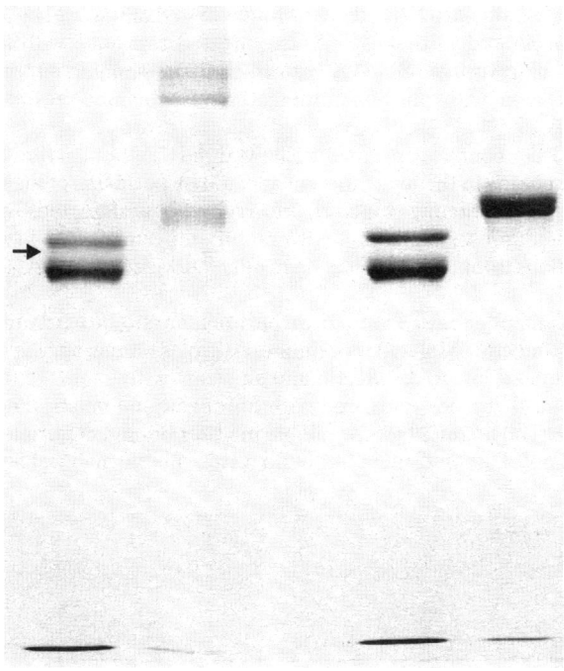

FIG. 6. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Analysis of the 200-kDal fragments and human plasma fibronectin used in the injection experiments. Lanes: 1 and 2, 200-kDal fragments and fibronectin without reduction; 3 and 4, the same samples after reduction with 2-mercaptoethanol. Arrow, migration position of myosin (200,000 daltons).

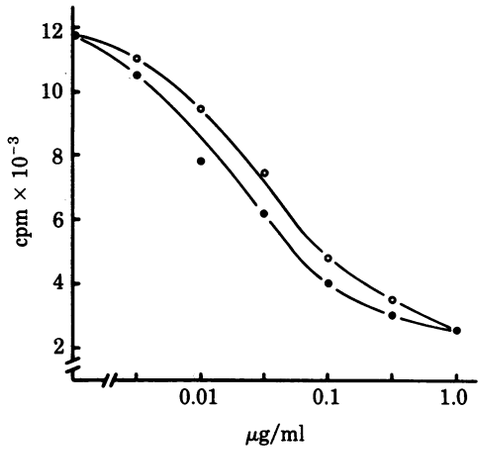

FIG. 7. Radioimmunoassay of human fibronectin. The binding of ^{125}I-labeled human plasma fibronectin to antifibronectin was inhibited by increasing concentrations of intact fibronectin (○) and 200-kDal fragments (●).

mouse tissues was indistinguishable from the distribution of the mouse's own fibronectin. That the presence of the injected fibronectin in the tissues was not a result of a simple presence of soluble fibronectin in the vascular and extravascular spaces is made unlikely by the results of several experiments.

(*i*) Perfusion of the livers did not noticeably alter the distribution or intensity of the staining for the injected fibronectin in the tissue.

(*ii*) Injected human IgG was not detected in tissues showing that the accumulation in tissues is not a characteristic generally associated with injection of foreign protein.

(*iii*) Fibronectin fragments that retain full immunological reactivity as well as major functional activities showed impaired capacity to become incorporated in tissues when injected into mice. In spite of blood levels similar to those obtained with whole fibronectin, the fragments, unlike fibronectin, were not present in the tubular areas of the kidney or in the sinusoidal areas of the liver. The staining found in the glomeruli and blood vessel walls may have been due to the trapping of some of the circulating material at these locations.

(*iv*) We have recently obtained additional evidence for the incorporation of plasma fibronectin in tissues by showing that injection of radiolabeled fibronectin from mouse plasma is followed by accumulation of radioactivity in tissues at levels exceeding those in plasma (unpublished results).

It is not known what makes fibronectin exist as an insoluble protein in tissues and as a soluble protein in plasma. An increasing body of evidence shows that the ability to become incorporated in an insoluble matrix is shared by the soluble forms of fibronectin. Fibronectin from fetal calf serum becomes incorporated in the matrix of cells cultured in its presence (20).

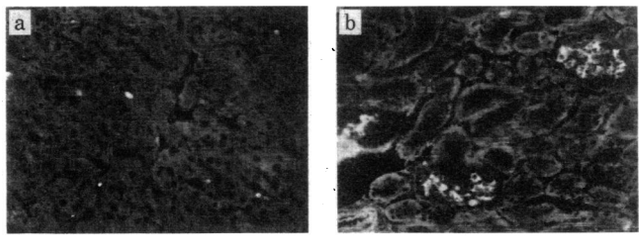

FIG. 8. Immunofluorescent staining for human fibronectin in the liver (*a*) and kidney (*b*) of a mouse injected with 200-kDal fibronectin fragments. Note absence of staining in the liver sinusoidal areas and around the tubular basement membranes of the kidney.

Similarly, fibronectin from culture media of transformed rat kidney cells, which does not form a matrix at the surface of these cells, does become incorporated in the matrix at the surface of these cells, does become incorporated in the matrix of normal fibroblasts (22). It has been suggested that interactions with other matrix components such as collagen and proteoglycans (14, 23) or crosslinking (24–26) could be important in determining whether fibronectin becomes insoluble or remains soluble. The present findings suggest that, whatever the mechanism behind the insolubilization of fibronectin, integrity of the molecule is essential for this to happen *in vivo*.

The 200-kDal fragments did not become incorporated in tissue matrices to an appreciable extent. This was in spite of the fact that they have retained the ability to interact with collagen and with glycosaminoglycans as shown by their binding to gelatin-Sepharose and heparin-Sepharose, respectively. These fragments also retain the ability to mediate cell attachment (unpublished data). The present results show that these activities are not sufficient for insolubilization in tissue matrix.

NH_2-Terminal sequences of the 200-kDal fragments show that they have lost the NH_2-terminal 27-kDal part of the polypeptide (unpublished data). The 27-kDal fragment is readily cleaved off by plasmin and trypsin and contains the site at which fibronectin is crosslinked to fibrin and collagen by blood coagulation Factor XIII (26). The interchain disulfide bond(s) is thought to be near the COOH terminus of the molecule (27). The 200-kDal fragments, when unreduced, behaved as single polypeptide chains on sodium dodecyl sulfate/polyacrylamide gels, indicating that they are truncated at this end of the polypeptide also (Fig. 9; see also refs. 2, 28, and 29 for discussion of the structure of fibronectin). The disulfide bonding, ability to become crosslinked by Factor XIII, and other possible functions associated with the parts absent from the 200-kDal fragments may be necessary for the incorporation of fibronectin into tissue stroma. Further experiments along these lines should be helpful in delineating the exact requirements for the insolubilization of fibronectin.

Our results demonstrate that plasma fibronectin can become insolubilized in tissues *in vivo*. The normal plasma level of fibronectin is about 300 μg/ml (30). In spite of the fact that the level of human fibronectin we achieved with our injection schedule corresponded to only about 25% of the normal level of circulating fibronectin, the staining for human fibronectin in the tissues was comparable to that observed with anti-mouse fibronectin. This strongly suggests that plasma fibronectin is in an equilibrium with tissue fibronectin and that the functions of these two forms of fibronectin are linked.

There are some differences in the carbohydrate chains of fibronectin from plasma and cultured fibroblasts. Plasma fibronectin does not contain appreciable amounts of fucose (31, 32),

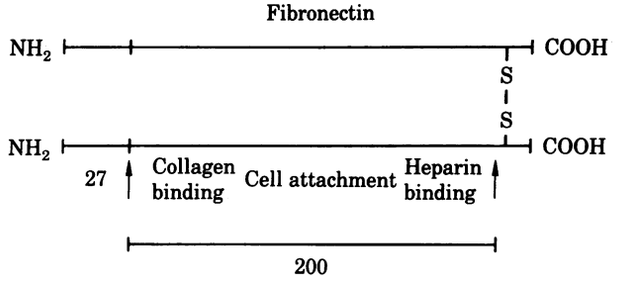

FIG. 9. A schematic representation of the structure of fibronectin and the 200-kDal fragments. Sizes shown are in kDal.

whereas this sugar is present in fibroblast-derived fibronectin (33, 34). Tissue fibronectin has not been studied. Determination of the carbohydrate composition of tissue fibronectin may allow conclusions regarding the extent to which plasma fibronectin contributes to the fibronectin in tissues.

The functions of plasma and tissue fibronectins are not well understood, but tissue fibronectin is likely to provide for normal cell attachment and may serve as an opsonin that enhances phagocytosis of tissue debris in tissue repair (see ref. 18). Our results suggest that administration of fibronectin could be helpful in facilitating such processes.

We thank Roland Carlsson for the liver perfusions and Drs. Eva Engvall and Edward G. Hayman for helpful discussions. This work was supported by Grant CA 27455 from the National Cancer Institute.

1. Mosher, D. F. (1980) *Prog. Hemostasis Thromb.* **5**, 111–151.
2. Ruoslahti, E., Hayman, E. G. & Engvall, E. (1981) *Collagen Res.* **1**, 95–128.
3. Klebe, R. J. (1974) *Nature (London)* **250**, 248–251.
4. Pearlstein, E. (1976) *Nature (London)* **262**, 497–500.
5. Grinnell, F., Hays, D. G. & Minter, D. (1977) *Exp. Cell Res.* **110**, 175–190.
6. Ruoslahti, E. & Hayman, E. G. (1979) *FEBS Lett.* **97**, 221–224.
7. Ruoslahti, E., Vaheri, A., Kuusela, P. & Linder, E. (1973) *Biochim. Biophys. Acta* **322**, 352–358.
8. Vuento, M., Wrann, M. & Ruoslahti, E. (1977) *FEBS Lett.* **82**, 227–231.
9. Birdwell, C. R., Brasier, A. R. & Taylor, L. A. (1980) *Biochem. Biophys. Res. Commun.* **97**, 574–581.
10. Balian, G., Crouch, E., Click, E. M., Carter, W. G. & Bornstein, P. (1979) *J. Supramol. Struct.* **12**, 505–516.
11. Ruoslahti, E., Engvall, E., Hayman, E. G. & Spiro, R. (1980) *Biochem. J.* **193**, 295–299.
12. Engvall, E. & Ruoslahti, E. (1977) *Int. J. Cancer* **20**, 1–5.
13. Stathakis, N. E. & Mosesson, M. W. (1977) *J. Clin. Invest.* **60**, 855–865.
14. Ruoslahti, E., Pekkala, A. & Engvall, E. (1979) *FEBS Lett.* **107**, 51–54.
15. Keski-Oja, J., Sen, A. & Todaro, G. J. (1980) *J. Cell Biol.* **85**, 527–533.
16. Yamada, K. M. & Kennedy, D. W. (1979) *J. Cell Biol.* **80**, 492–498.
17. Blumenstock, F., Weber, P. & Saba, T. M. (1977) *J. Biol. Chem.* **252**, 7156–7162.
18. Saba, T. M. & Jaffe, E. (1980) *Am. J. Med.* **68**, 577–594.
19. Ruoslahti, E., Vuento, M. & Engvall, E. (1978) *Biochim. Biophys. Acta* **534**, 210–218.
20. Hayman, E. G. & Ruoslahti, E. (1979) *J. Cell Biol.* **83**, 255–259.
21. Köhler, G. & Milstein, C. (1975) *Nature (London)* **256**, 495–497.
22. Hayman, E. G., Engvall, E. & Ruoslahti, E. (1980) *J. Cell Biol.*, in press.
23. Jilek, F. & Hörmann, H. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 597–603.
24. Keski-Oja, J., Mosher, D. F. & Vaheri, A. (1976) *Cell* **9**, 29–35.
25. Hynes, R. O. & Destree, A. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 2855–2859.
26. Mosher, D. F., Schad, P. E. & Vann, J. M. (1980) *J. Biol. Chem.* **255**, 1181–1188.
27. Chen, A. B., Amrani, D. L. & Mosesson, M. W. (1977) *Biochim. Biophys. Acta* **493**, 310–322.
28. Wagner, D. D. & Hynes, R. O. (1980) *J. Biol. Chem.* **255**, 4304–4312.
29. Sekiguchi, K. & Hakomori, S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2661–2665.
30. Mosesson, M. W. & Umfleet, R. A. (1970) *J. Biol. Chem.* **245**, 5728–5736.
31. Wrann, M. (1978) *Biochem. Biophys. Res. Commun.* **84**, 269–274.
32. Takasaki, S., Yamashita, K., Suzuki, K., Iwanaga, S. & Kobata, A. (1979) *J. Biol. Chem.* **254**, 8548–8553.
33. Carter, W. G. & Hakomori, S. (1979) *Biochemistry* **18**, 730–738.
34. Fukuda, M. & Hakomori, S. (1979) *J. Biol. Chem.* **254**, 5451–5457.