Vol. 100, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 15, 1981 Pages 275-282

FIBRONECTIN AND COLLAGEN OF CULTURED SKIN FIBROBLASTS IN DIABETES MELLITUS*

Barbara D. Smith† and Cynthia K. Silbert

Connective Tissue-Aging Research Laboratory
Veterans Administration Outpatient Clinic and
Veterans Administration Medical Center, Boston, MA 02108

Received February 26,1981

Summary

The incorporation of [14C]proline and [14C]glycine into total protein, collagen and fibronectin was investigated in cultures of human skin fibroblasts obtained from normal individuals and from patients with Type I, insulin-dependent diabetes mellitus. Proteins from the media and cell layer were separated using polyacrylamide SDS gel electrophoresis. Cells derived from diabetics incorporated more radioactivity into total protein, collagen, and fibronectin. The radioactivity in collagen increased more than fibronectin, producing a lower fibronectin to collagen ratio in diabetic fibroblasts. With increasing passage in culture, cell lines from normal and diabetic individuals showed an increased fibronectin to collagen ratio in media. This ratio increased more in cell lines from normals than from diabetics. This study provides additional evidence for altered production of intercellular matrix by cultured fibroblasts in diabetes mellitus.

Introduction

Diabetes mellitus is associated with thickening of collagen-rich basement membranes and with an increased incidence of degenerative changes of arteries and nerves. Since diabetes may have a genetic component, we (1) and others (2-6) have utilized cultures of skin fibroblasts derived from individuals with diabetes to study potential abnormalities in growth and biochemical parameters, in particular, changes in connective tissue or intercellular compounds. We have described an increased proportion of the glycosaminoglycan, heparan sulfate, in the media of fibroblast cultures from diabetics (1). An increase in protein and collagen in media of similar cultures has been described by others (2,3) as well as a decrease

*This investigation was supported by VA Medical Research Service.

† Requests for reprints should be addressed to B.D. Smith, VA Outpatient Clinic, 17 Court Street, Boston, MA 02108.

0006-291X/81/090275-08$01.00/0

in a presumed procollagen protein band which migrated ahead of collagen β chains (3).

We have now studied the incorporation of radioactive proline and glycine into proteins by cultures of skin fibroblasts obtained from patients with insulin-dependent diabetes mellitus and age-matched normal subjects. We have specifically identified the protein migrating more slowly than the collagen β chains as fibronectin. We have also noted changes in the fibronectin to collagen ratio between skin fibroblasts of normal and diabetics during in vitro aging.

Materials and Methods

Fibroblasts were grown, as previously described (7), from skin punch biopsies obtained from three insulin-dependent non-obese individuals and two normals, 20-25 years old. Cells banked in liquid nitrogen at 3rd to 4th passage were thawed and grown in Dulbecco's media containing 10% fetal calf serum for experimental studies. Between 7th and 14th passage, cell lines were grown to confluence in T25 flasks, then labeled for 24 hours with [14C]proline (2μc/ml) and [14C]glycine (2μc/ml) in Dulbecco's media without glutamine or serum, but with 50μg/ml β-aminoproprionitrile and ascorbic acid. Cell number was determined in a duplicate flask using a Coulter Counter. Media were removed at 24 hours, and a concentrated solution of EDTA, Tris, and protease inhibitors was added to yield a final concentration of 10μM phenylmethylsulfonyl fluoride (PMSF), 1mM parachloromercurobenzoate (PCMB), 20mM EDTA, 50mM Tris, pH 7.4. The washed cell layers were homogenized with a Polytron homogenizer and extracted in 150mM NaCl, 0.1% deoxycholate, 10μM PMSF, 1mM PCMB, 20mM EDTA, 20mM CAPS, pH 11 buffer.

Protein in the media and cell layer extracts was precipitated with 4 volumes of ethanol for 16 hours at -20°C and centrifuged at 9,500xg for 30 minutes. The precipitated proteins were suspended in 5mM Hepes and aliquoted for the appropriate experiments. Radioactivity was determined using a Searle liquid scintillation counter.

Aliquots were prepared for electrophoresis by heating samples for 5 minutes at 90°C in electrophoresis sample buffer (8) with dithiothreitol (DTT) and electrophoresed on 5% or 7.5% polyacrylamide sodium dodecyl sulfate (SDS) gel. In some cases, samples were treated with purified bacterial collagenase (Advanced Biofactors, Form III) for 16 hrs before electrophoresis. Radioactive chicken calvaria procollagen and fibronectin (gift of M. Perkins) were used as standards. The gels were stained with Coomassie Brilliant Blue R-250, destained with 5% methanol and 7% acetic acid, enhanced by treating with Autofluor (National Diagnostics) for 1 hour, dried, and exposed to X-ray film (9). The fluorograms were scanned at 540nm using a Beckman model 35 spectrophotometer adapted with a gel scanning attachment, and the area under the curves was calculated. The radioactivity of gel strips was determined by cutting gels into 1mm strips, digesting with Protosol (New England Nuclear) and suspending in Aquassure (New England Nuclear) for liquid scintillation counting. Greater than 80% of the radioactivity was recovered by this method. The scan areas (540nm) were directly proportional to counts per minute in each protein band. The amount of collagen present was quantitated in representative experiments by washing the ethanol precipitates with 5% TCA to remove any

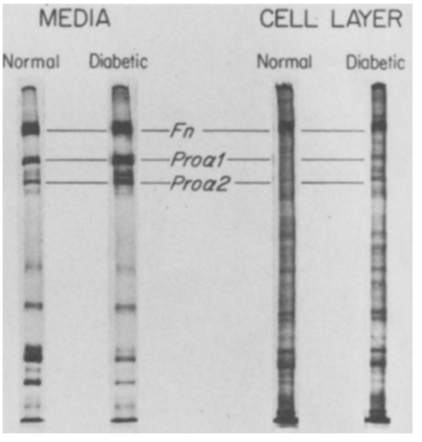


Figure 1: A 7.5% polyacrylamide SDS gel electrophoresis fluorogram of [14C]proline and glycine labeled proteins from skin fibroblast media and from cell layer of both normal (passage 10) and diabetic (passage 9) individuals. The position of standard fibronectin (Fn) and procollagen chains (pro α1 and pro α2) electrophoresed in adjacent lanes are indicated on the figure.

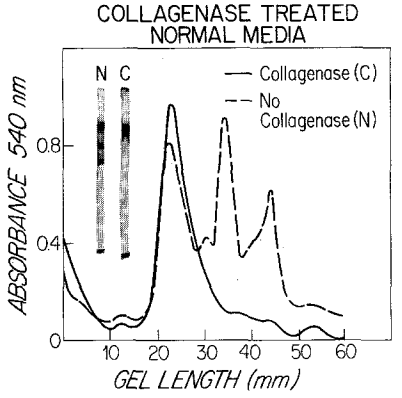
remaining free counts, followed by collagenase assays using the method of Peterkofsky (10).

Fibronectin was identified using rabbit anti-fibronectin antibody (Collaborative Research, or a gift from L.B. Chen). Aliquots of media fraction before precipitation with ethanol were incubated with 20μl of anti-fibronectin antibody or non-immune serum for 30 minutes, followed by an incubation for 16 hours at 44°C with 25μl of anti-rabbit IgG. The precipitate was washed three times with PBS and 1% triton X-100, heated to 90°C for 5 minutes in electrophoresis sample buffer and electrophoresed on a 7.5% polyacrylamide gel. The precipitation and electrophoresis were also performed on standard fibronectin (Collaborative Research) and radioactive fibronectin from guinea pig fibroblast media (a gift from M. Perkins).

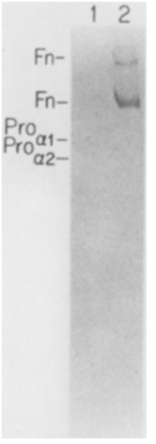
Results

In four separate experiments, the incorporation of radioactivity into ethanol-precipitated media proteins and cell layer proteins during a 24 hour pulse was 2 - 2.5 times greater in the cells from diabetics than in the cells from controls normalized for cell number. The collagenase-sensitive protein was increased 2 to 6 fold (mean 3.9) for diabetic cells compared to normal cell lines.

The proteins from both media and cell layer were electrophoresed on polyacrylamide SDS gels after reduction with DTT, and run with standard chick calvaria procollagen and fibronectin (Figure 1). A similar number of



(2)



(3)

Figure 2: A 5% polyacrylamide SDS gel electrophoresis fluorogram and densitometric scan of normal-fibroblast media proteins (passage 8) with (—— C) and without (--- N) collagenase.

Figure 3: A 7.5% polyacrylamide SDS gel electrophoresis fluorogram of normal fibroblast media precipitated with fibronectin antibody (lane 2) and anti-rabbit IgG. Lane 1 is a control precipitation with non-immune serum and anti-rabbit IgG. Position of standard fibronectin (Fn) and procollagen chains (pro α1 and pro α2) electrophoresed in adjacent lanes are indicated. Samples were not completely reduced with DTT.

counts was applied to each gel. The three high molecular weight bands, comigrating with procollagen α chains and with fibronectin, were darker in diabetic cell media and in cell layer fractions. Coomassie Blue staining was similarly increased for the same three high molecular weight bands. Two lower weight proteins appeared to be significantly decreased in the media fraction of diabetic cell lines.

Further identification of the three high molecular weight protein bands was carried out on media of both diabetic and control cultures. The fluorogram gel scans with and without collagenase treatment (Figure 2) indicates that the first protein band migrating with fibronectin standard was not collagenase sensitive and therefore, was not a procollagen dimer as previously suggested (3). The other two bands were indeed collagenous. Electrophoresis of the precipitate formed with fibronectin antibody (Figure 3) confirmed that the first radioactive band was fibronectin.

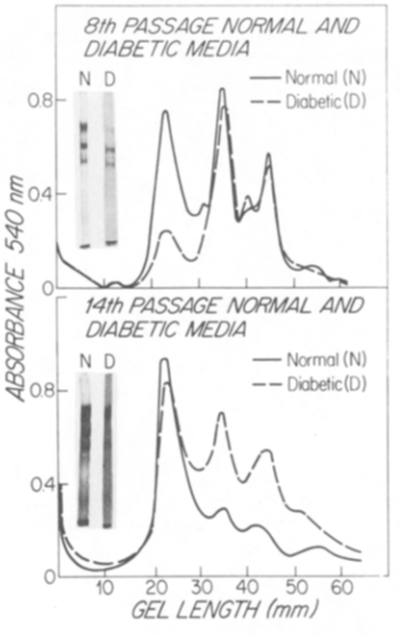


Figure 4: A 5% polyacrylamide SDS gel electrophoresis fluorgram and densitometric scan of normal (—— N) and diabetic (--- D) media proteins from 8th and 14th passage cells. The same number of counts were applied to each lane.

When fluorogram gel scans of radioactive proteins produced by normal and diabetic individuals were compared (Figure 4), it was clear that the ratio of fibronectin to collagen (F/C) was much lower in diabetic cell lines for both early (8th) and late (14th) passage. Also, both diabetic and normal cultures had an increased F/C ratio at later passages. In Table I, the F/C ratios found in media are given for three different diabetic patients and two controls. There was a marked increase in F/C ratio with later passages by the cell lines derived from normals while diabetic cell lines showed a less marked change in ratio during *in vitro* aging.

Discussion

Fibroblasts derived from insulin dependent diabetics incorporated more than twice the amount of radioactive proline and glycine into total protein in both media and cell layers than was incorporated by cells from age-matched controls. Perhaps more important than the synthesis of total pro-

TABLE I
MEDIA FIBRONECTIN/COLLAGEN RATIOS

Passage #	Normal	Diabetic
7	0.54 (P)*	0.47 (J)
	--	0.35 (W)
8	0.9 (B)	0.16 (Q)
	0.9 (B)	--
10	1.4 (B)	0.39 (J)
	1.9 (P)	--
14	3.3 (B)	1.25 (Q)

* The letter in parentheses refers to individual patient's cell line.

teins was that the proportion of proteins accumulated in the media differed in cultures of diabetic cells. Without exception, each fibroblast cell line from diabetics had a lower media fibronectin to collagen ratio. This occured because the incorporation of radioactivity into collagen was increased more than total protein or fibronectin. This consistent finding is of particular interest since this could not be explained by differences in pool sizes between cell lines. Although Branson et al. (6) did not comment upon it, these authors had a similar finding of a lowered fibronectin to collagen ratio in diabetic cells which were pulsed for several hours immediately following trypsinization. The collagenase-resistant protein band which we have identified as fibronectin was previously reported to be decreased in media of diabetic cells (3). However, this band was described as a procollagen dimer and was not recognized to be fibronectin.

Although diabetic fibroblasts in general showed a greater incorporation of radioactivity into proteins, two low molecular weight proteins found in normal fibroblasts were decreased in two diabetic cell lines and were absent in a third diabetic cell line. These proteins are currently being investigated No clinical differences between the several diabetic patients were apparent.

According to several investigators, normal cells have a finite life span which is inversely proportional to an individual's age (11). For this reason, cell culture has been used as a model for the cellular aging process. The increase in fibronectin to collagen ratio with passage level, found in this study, might reflect changes in connective tissue during aging in vivo. Fibronectin binds to collagen near the mammalian collagenase-sensitive site (12). Thus, more fibronectin formation with age could inhibit collagen breakdown and could make collagen less soluble. Both these properties have been repeatedly observed for "aged" collagen.

Diabetes is often referred to as a disease of accelerated aging (13). In support of this theory, it has been reported that human skin fibroblasts from type I diabetics have a reduced growth potential (5) and fibroblasts from presumed "pre-diabetics" have a reduced plating efficiency compared to normal controls (4). Collagen becomes both less soluble and less susceptible to collagenase at an earlier age in diabetes (13). If the process of cellular aging were to be accelerated in diabetes, it might be expected that the fibronectin to collagen ratio at each passage level would reflect the changes that are found in aging cells. Thus, the ratio of fibronectin to collagen would be higher for diabetic cells than for normal cells. The reverse was observed, and the ratio did not increase as significantly with age, suggesting that aging and diabetes are separate, distinct processes.

Acknowledgements

We thank Ms. Ann Mahoney and Mr. Alfred Pistorio for their technical assistance.

References

1. Silbert, C.K. and Kleinman, H.K. (1979) Diabetes 28:61-64
2. Rowe, D.W., Starman, B.J., Fujimoto, W.Y. and Williams, R.H. (1977) Diabetes 26:284-290.
3. Kohn, R.R. and Hensse, S. (1977) Biochem. Biophys. Res. Commun. 76:765-771.
4. Goldstein, S., Littlefield, J.W. and Soeldner, J.S. (1969) Proc. Natl. Acad. Sci. U.S.A. 64:155-160.

5. Goldstein, S., Moerman, E.J., Soeldner, J.S., Gleason, R.E. and Barnett, D.M. (1978) Science 199:781-782.
6. Branson, R.E., Lembach, K.J. and Cunningham, L.W. (1980) In Vitro 16:159-167.
7. Kleinman, H.K., Silbert, J.E. and Silbert, C.K. (1975) Connect. Tiss. Res. 4:17-23.
8. Laemmli, U.K. (1970) Nature 227:680-685.
9. Laskey, R.A. and Mills. A.D. (1975) Eur. J. Biochem. 56:335-341.
10. Peterkofsky, B. and Diegelmann, R. (1971) Biochemistry 10:988-994.
11. Hayflick, L. and Moorhead, P.S. (1961) Exp. Cell Res. 25:585-621.
12. Kleinman, H.K., McGoodwin, E.B., Martin, G.R., Klebe, R.J., Fietzek, P.P. and Woolley, D.E. (1978) J. Biol. Chem. 253:5642-5646.
13. Kohn, R.R. and Hamlin, C.R. (1978) Birth Defects: Original Article Series 14:387-401.