IMMUNOHISTOCHEMICAL LOCALIZATION OF FIBRONECTIN IN AORTIC INTIMA OF RAT

YOSHITERU KATSUKURA, TAKESHI TSUCHIYA, NOBUYUKI ABE
AND NOBUYUKI WATABE*

*Department of Radiology, Tohoku University, School of Medicine, Sendai 980
and Department of Radiology*, Tohoku Teishin Hospital, Sendai 980*

Received for publication April 19, 1982 and in revised form August 3, 1982

The localization of fibronectin in the aortic intima from 2, 4 and 8 months old SHR were observed with immunoperoxidase technique. Acid polysaccharide stainings were consistently performed on the serial sections of the same specimen.

In 2 months old rats, aortic intima showed normal structure arrangement. In such cases, fibronectin localized linearly in the basement membrane underlying epithelium. The intima from rats of over 4 months of age tended to thicken with fibrin-like and fibrous substances, and injury to epithelium was often observed. In these cases, reaction products appeared to be deposited from plasma fibronectin and by secretion of migrated cells from the media. Acid polysaccharides positive area was similar to the portions of fibronectin deposits. Some discussion was held on the pathogenesis of the primary stage of arteriosclerosis correlated with fibronectin mobilization.

Intimal injury is the most significant lesion in the primary stage of arteriosclerosis. It is thought that platelets and fibrinogen, the proliferative factors of smooth muscle cells, infiltrate into the intimal matrix and there they induce smooth muscle cells to migrate (2, 11). Pathological degeneration of media is based on calcium deposition or lipid infiltration into the medial layer and coacervation of elastin peptides are inhibited by these pathological changes (4, 5, 13).

Fibronectin is one of the glycoproteins distributed on the cell surface. It has a high affinity to collagen, its derivatives and sulfopolysaccharides. Fibronectin in normal and some pathological conditions has been reported with histo- and cytochemical studies (1, 6).

This study was undertaken to focus on the distribution of fibronectin in relation to the distribution of acid mucopolysaccharides under normal and abnormal status, such as intimal thickening in the primary stage of arteriosclerosis.

MATERIALS AND METHODS

1. Experimental animals and tissue preparation

Three male SHR (spontaneously hypertensive rat) at 2, 4 and 8 months of age were used in this study, respectively (9). Under nembutal anesthesia, animals

were exsanguinated and then perfused with 10% formalin. All specimens of superior aorta were refixed with FP-fixative at 4°C overnight and embedded in paraffin. This fixative was composed of saturated solution of picric acid 75 ml and formalin 25 ml. Two μ m thick paraffin sections were prepared for the histo- and immunohistochemical stainings.

2. Staining procedures

Hematoxylin-eosin, sudan-hematoxylin and periodic acid Schiff-alcian blue stain (pH 2.5) were made for overall light microscopic observations. Diamine-alcian blue stain was used to demonstrate sulfopolysaccharides (12).

Direct immunoperoxidase technique for fibronectin was performed as follows; The thin sections were kept on glass slides at 37°C overnight, deparaffinized and washed in 2 changes of 0.05 M Tris-saline, pH 7.6, for 5 min, then incubated in the peroxidase-labeled anti-human fibronectin antiserum for 25 min at a 1/20 dilution.

The antibody used for the direct immunoperoxidase method was rabbit anti-human fibronectin HRPO conjugate developed by Nakane and Kawaoi (8) (HRPO, horseradish peroxidase, Type VI, Sigma Co., Ltd. and rabbit anti-human fibronectin antiserum from Collaborative Res. Inc.). Three washes with Tris-saline at 4°C were carried out for 30 min, followed by immersion in 0.05% 3, 3'-diaminobenzidine in Tris-saline buffer with 0.01% H_2O_2 for 15 min (3, 3'-diaminobenzidine tetrahydrochloride, Sigma Co., Ltd.). After washing in 3 changes of Tris-saline buffer for 5 min and where necessary, the cell nuclei were stained with Mayer's hematoxylin. The specificity of this antiserum was evaluated for cross reactions with rat fibronectin (rat fibronectin, Calbiochem-Behring).

For the control, the following experiments were carried out instead of the antiserum; a, with normal rabbit serum labeled with HRPO and b, with anti human fibronectin antiserum which was adsorbed with human fibronectin (human fibronectin, Collaborative Res. Inc.).

RESULTS AND DISCUSSION

After incubation of deparaffinized sections with peroxidase-labeled rabbit anti-human fibronectin antiserum, specific immunoperoxidase reaction was observed in the aortic intima and adventitia of SHR. The elastic fibers in the aorta of SHR were not stained by this procedure. In rats of 2 months of age, fibronectin codistributed in the basement membrane underlying epithelium (Figs. 1, 2). The reaction products for fibronectin in the basement membrane underlying epithelium suggested that this glycoprotein is a structural component of the basement membrane and that it may be, at least in part, synthesized and deposited by the intimal epithelium. Diamine-alcian blue staining of these sections revealed similar sites of fibronectin distribution (Figs. 3, 4).

In rats of the 4 month group, the amount of fibronectin reduced markedly as compared with rats of the 2 month group (Figs. 5, 6). This difference may be related to a decrease in the amount of loose connective tissue during cell aging or injury (6). In the thickened intima (fibromuscular thickening) from this group, fibronectin was codistributed diffusely with fibrillar connective tissue components (Fig. 7). In this age group, fibronectin appeared to be deposited from serum and

FIGS. 1 and 2. Aorta from 2 months old SHR. The tissue was fixed in FP solution at 4°C overnight. The preparation was incubated with peroxidase-labeled anti-fibronectin antiserum. Staining was carried out by the direct immunoperoxidase method and shows immuno-reactive products underlying epithelium (arrows) and of adventitia (a). $\times 360$

excreted from the migrated cells. Collagen fibers might have been transferred to fragmented or denatured ones by serological factors at this stage.

Pericellular fibronectin fibrils were lost in rats of the 4 month group. According to Mosher and Vaheri (7), fibronectin was lost after exposure for 4–20 hr to thrombin (1–8 U/ml). Loss of fibronectin fibrils did not occur if thrombin was inhibited by PMSF (phenyl-methyl-sulfonyl fluoride), α -2-macroglobulin or hirudin. According to their contentions, it seemed that rats of the 2 month group had a normal composition of serum, which contained inhibitors of thrombin, and hence only low levels of thrombin activity also stimulated fibronectin production but did not cause loss of pericellular fibronectin fibrils. In rats of the 4 and 8

FIGS. 3 and 4. Diamine-alcian blue stain of the series sections of Figs. 1 and 2. Diamine-alcian blue positive portions coincide with immuno-reactive area. Adventitia show not so positive staining. $\times 360$

month group, respectively, the deposition of lipids and protein in the intima was recognized and it suggested that these substances evoked smooth muscle cell migration or fibroblast proliferation and collagen fiber production and led to fibromuscular thickening of the intima. Serum thrombin in these groups was supposed to contain a higher concentration of thrombin than that of the 2 month group. The thickened intima of rats in the 8 month group were filled with fibrin-like substances (Figs. 8–10). These areas usually tend to be calcified matrix (3). The localization of fibronectin was very similar to that of alcian blue positive portions (Figs. 11–13). In this group, epithelial surface were markedly ruptured.

The biological roles of fibronectin *in vivo*, however, are basically still unknown. In this study, changes of fibronectin distribution with age seemed to be based on

the epithelial cell damage and followed on plasma penetration and smooth muscle cells migration from medial layer. In addition, collagen might be degraded during the above changes and remodeled at sites of tissue injury. Plasma fibronectin might be incorporated and deposited into this lesional area. Denatured collagen was found to have an even higher capacity per weight to bind fibronectin than normal collagen. Of course, neither could any inhibition be obtained with purified mucopolysaccharides, such as heparin, hyaluronic acid or chondroitin sulfate A, B and C (1). These findings and conceptions suggest that collagen and collagen-derived peptides might function as chemotactic stimuli for fibroblast *in vivo* and attract these cells to the lesional area (10).

In the above observations, it is assumed that platelets deposit on the complex components, such as plasma fibronectin, acid mucopolysaccharides, calcium salts and denatured collagen fibers in thickened layer and pathogenesis of arteriosclerosis is promptly accelerated with these complicated substances.

REFERENCES

1. Engvall, E. and Rouslahti, E.: Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. *Int. J. Cancer*. 20; 1-5, 1977.
2. Ishida, T., Watanabe, K. and Tanaka, K.: Effects of fibrin, fibrinogen and its derivatives to proliferation of smooth muscle cells in culture. *Arteriosclerosis* 8; 605-615, 1981 (*in Japanese*).
3. Katsukura, Y., Tsuchiya, T., Abe, N., Miyachi, H., Watabe, N. and Hoshino, F.: Some aspects on systemic fibrosis. *Jpn. J. Lymphol.* 2; 15-19, 1979 (*in Japanese*).
4. Kramsch, D. M., Franzblau, C. and Hollander, W.: The protein and lipid composition of arterial elastin and its relationship to lipid accumulation in the atherosclerotic plaque. *J. Clin. Invest.* 50; 1666-1677, 1971.
5. Kramsch, D. M. and Hollander, W.: The interaction of serum and arterial lipoproteins with elastin of the arterial intima and its role in the lipid accumulation in atherosclerotic plaques. *J. Clin. Invest.* 52; 236-247, 1973.
6. Linder, E., Stenman, S., Lehto, V. P. and Vaheri, A.: Distribution of fibronectin in human tissues and relationship to other connective components. *Ann. New York Acad. Sci.* 312; 151-159, 1978.
7. Mosher, D. F. and Vaheri, A.: Thrombin stimulates the production and release of a major surface-associated glycoprotein (FN) in cultures of human fibroblast. *Exp. Cell Res.* 112; 323-334, 1978.
8. Nakane, P. K. and Kawaoi, A.: Peroxidase-labeled antibody. A new method of conjugation. *J. Histochem. Cytochem.* 22; 1084-1091, 1974.
9. Okamoto, K. and Aoki, K.: Development of a strain of spontaneously hypertensive rat. *Jpn. Circulation J.* 27; 282-293, 1963.
10. Postlethwaite, A. E., Seyer, J. M. and Kang, A. H.: Chemotactic attraction of human fibro-

Figs. 5-7. Immunoperoxidase reactions of 4 months old rats. Reaction products are markedly reduced as compared with Figs. 1 and 2. Fig. 7 shows marked fibromuscular thickening in the intima and destructed epithelia. Fig. 5. $\times 360$, Figs. 6 and 7. $\times 720$

Figs. 8-10. Immunoperoxidase reactions of 8 months old rats. Thickened intima with fibrin-like and fibrous substances (S) are shown. The localization of reaction products are diffusible as compared with the specimens from 2 months old rats. Epithelium are markedly ruptured. $\times 720$

Figs. 11-13. Diamine-alcian blue stain of the serial sections of Figs. 8-10. Diamine-alcian blue positive portions are shown consistent with fibronectin deposits. $\times 720$

- blasts to Type I, II, III collagens and collagen-derived peptides. *Proc. Natl. Acad. Sci. USA*. 75; 871-875, 1978.
11. Rutherford, R. B. and Ross, R.: Platelets factors stimulate fibroblasts and smooth muscle cells quiescent in plasma serum to proliferate. *J. Cell Biol.* 69; 196-203, 1976.
 12. Spicer, S. S.: Diamine methods for differentiating mucosubstances histochemically. *J. Histochem. Cytochem.* 13; 211-234, 1965.
 13. Urry, D. W.: Molecular perspectives of vascular wall structure and disease: The elastic component. *Perspect. Biol. Med.* 21; 265-295, 1978.