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Changes in the Collagen Binding Property of Human Plasma Fibronectin with Aging

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ABSTRACT. We investigated changes in quality of plasma fibronectin with aging, especially in the interaction of fibronectin with collagen, using an enzyme-linked immunosorbent assay. When fibronectin isolated from the plasma of young people was compared with that from old people, the latter was found to bind poorly to gelatin, Type I and Type III collagens. No differences in molecular weight were detected between the two types of fibronectin by SDS-polyacrylamide gel electrophoresis.

Key words : Fibronectin — Collagen — Aging

Some studies have indicated that changes occur in the quantity of fibronectin from human fibroblasts with cellular aging.[1-4] Cell surface fibronectin has been observed to decrease in quantity with cellular aging.[1,3,4] The amount of fibronectin per cell or per cell protein released into culture medium has also been observed to increase with cellular aging.[3,4] Recently Chandrasekhar *et al.*[5] reported an age specific defect in the biological activity of human fibroblast fibronectin, especially in the interaction of fibronectin with collagen. We believe that contradictory results concerning changes in the quantity of cell surface fibronectin and released fibronectin with cellular aging are caused by defects in aged cellular fibronectin.[2,5]

Plasma fibronectin concentration, on the other hand, increases with aging according to Eriksen *et al.*[6] and Labat-Robert *et al.*[7] Labat-Robert *et al.* speculated that defects in aged cellular fibronectin are compensated for an overproduction of plasma fibronectin in the aging human body. We believe, however, that plasma fibronectin might also exhibit defects with aging. Therefore, we decided to investigate changes in the quality of plasma fibronectin with aging, especially in the interaction of fibronectin with collagen, with an enzyme linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

1. *Collection of samples*

Plasma samples were obtained from 10 young volunteers in their twenties and from 10 elderly volunteers in their eighties. Blood was collected in sodium

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citrate and plasma was separated by centrifuging at 2,000 rpm for 15 minutes at temperature. It was then stored at −20°C until use and thawed at 37°C. This storage has been shown not to alter plasma fibronectin values.[6,7]

2. *Purification of fibronectin*

Plasma fibronectin was purified essentially according to the method of Engvall and Yamada,[8,9] using a cyanobromide activated gelatin-sepharose 4B column (Pharmacia, Tokyo, Japan). The column was washed with 0.05 M Tris buffer (pH 7.4) containing 0.025 M 6-aminohexanoic acid, plasma samples were filtered through, and unbound plasma proteins were removed with 0.05 M Tris buffer containing 0.025 M 6-aminohexanoic acid. Fibronectin was eluted with 8 M urea in 0.05 M Tris buffer, as shown in Figure 1. The eluted fibronectin was dialysed with 0.01 M cyclohexylaminopropane-sulfonic acid (CAPS) buffer (pH 11.0). Purified plasma fibronectin isolated from the plasma of young people (FN-Y) and from the plasma of old people (FN-O) were quantified as described earlier using and ELISA technique.[3] Then the concentrations of both fibronectins were equalized with 0.01 M CAPS buffer (85 μg/ml). Both purified fibronectins were stored at −20°C until use then thawed at 37°C.

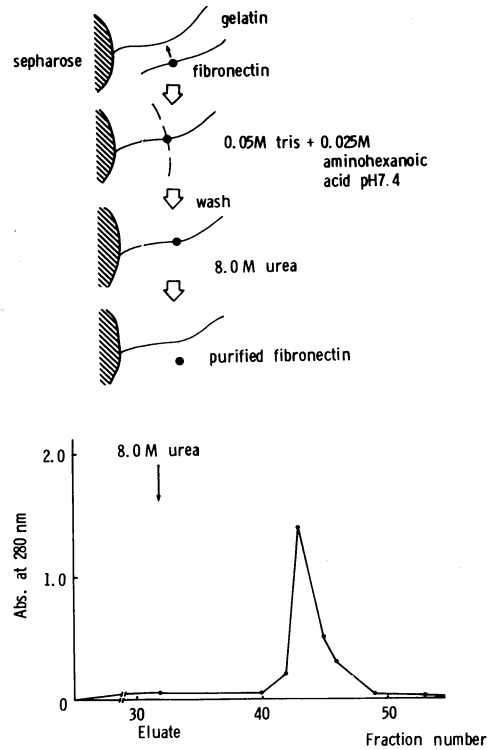

Fig. 1. Purification of plasma fibronectin by a gelatin-sepharose 4B column.

3. *SDS-polyacrylamide gel electrophoresis*

Discontinuous gel electrophoresis with a 3% stacking gel and a 5% resolving gel was performed in a Tris-buffered system according to the method of Laemmli.[10] 0.05 M Tris-glycine buffer (pH 8.5) was used as the electrode buffer. Samples (plasma fibronectin) were mixed with an SDS mixture (1 M Tris-HCl (pH 7.0), 5 μl; 20% SDS, 10 μl; 7% Bromphenol blue, 0.5 μl; Glycerol, 10 μl ; Mercaptoethanol, 5 μl) at a 7:3 ratio and were boiled for 1.5 minutes. Electrophoresis was continued for 5 hours at 20 mA. For staining, the gel was soaked in a staining solution (0.1% Coomsassie brilliant blue, 10% Acetic acid, 25% Methanol). Then it was soaked in a distaining solution (10% Acetic acid, 25% Methanol).

4. *Enzyme linked immunosorbent assay (ELISA)*

1) Interaction of human plasma fibronectin with substrate adsorbed collagen using an ELISA (Direct binding assay)

For the binding assay, 96-well flat-bottomed microtiter plates (Dynatech, Plochingen, West Germany) were coated with either gelatin (Nakarai, Kyoto, Japan) or collagens. Before coating, Type III collagen (Sigma, St. Louis, USA) was made soluble in 0.01 M acetic acid. This solubilized Type III collagen

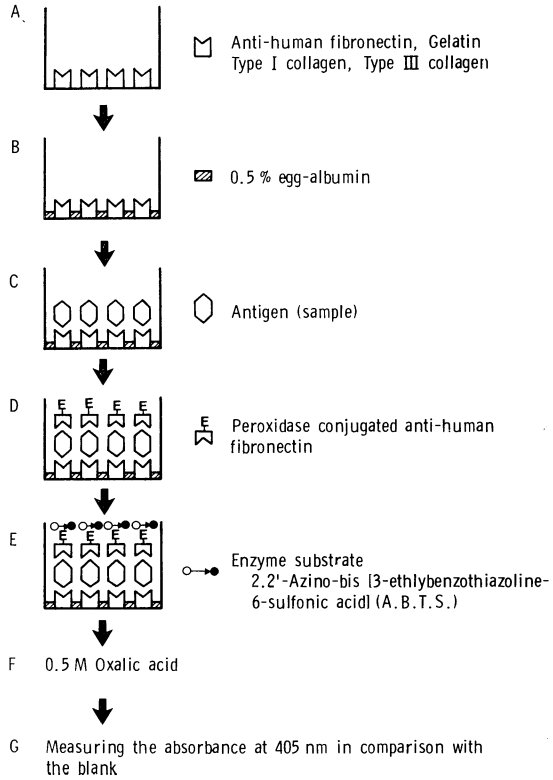

Fig. 2. Diagram of ELISA (Direct binding assay).

was diluted into a carbonate buffer (pH 9.6) at a final concentration of 10 μg/ml. Gelatin and Type I collagen (Koken, Tokyo, Japan) were diluted into a carbonate buffer at a final concentration of 10 μg/ml and 300 μg/ml, respectively. 100 μl of these proteins were added to the wells of the flat-bottomed microtiter plates (Fig. 2-A), and they were incubated at 37°C for 2 hours. The plates were then washed three times with phosphate buffererd saline containing 0.05% Tween 20 (PBS/T). Next, egg albumin was diluted with 0.01 M carbonate buffer (pH 9.6) at a final concentration of 0.5%, and 100 μl of the solution was added to each well and allowed to adsorb overnight (Fig. 2-B) at 4°C. Thereafter, the plates were washed three times with PBS/T. Samples (FN-Y, FN-O) were diluted with PBS, and 100 μl of each sample was added to each well (Fig. 2-C), and they were incubated at 37°C for 30 minutes. After the plates were washed again three times with PBS/T, 100 μl of peroxidase-conjugated antihuman fibronectin (Cappel Lab. Westchester, NY) at a 1:1000 dilution with PBS was added to the wells (Fig. 2-D) and they were incubated at 37°C for 30 minutes. The plates were then washed three times with PBS/T. Then 100 μl of 2.2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Medical Biological Lab., Nagoya, Japan), the enzyme substrate for Horse Radish Peroxidase, was added to each well (Fig. 2-E), and they were incubated again at 37°C for 30 minutes. ABTS becomes a radical cation with Horse Radish Peroxidase and produces a blue-green color. The enzyme reaction was stopped with 50 μl of 0.5 M oxalic acid (Fig. 2-F). The absorbance of the products of the enzyme reaction was measured using a micro ELISA autoreader, which reads the absorbance of the solution within the microtiter wells.

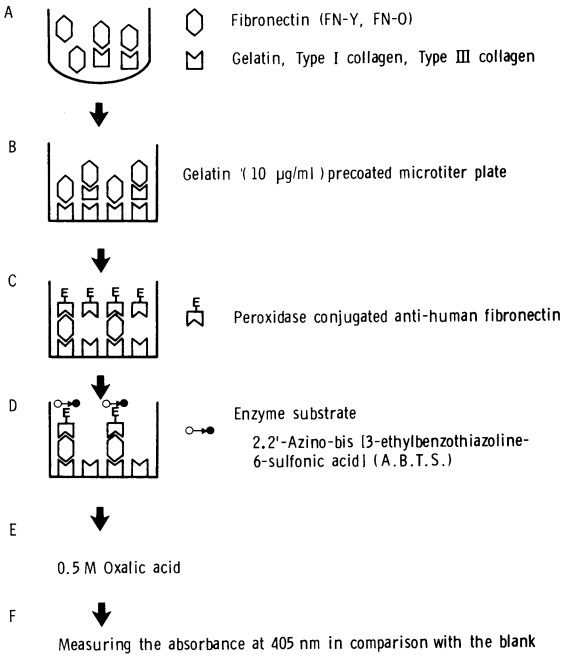

Fig. 3. Diagram of competitive inhibition ELISA.

2) Interaction of human plasma fibronectin with solubilized collagens using a competitive inhibition ELISA

For the inhibition assay, collagens (or gelatin) and fibronectin solution were diluted with PBS. The two kinds of samples of human plasma fibronectin (FN-Y, FN-O) were diluted at a final concentration of 8.5 μg/ml. Type I collagen, Type III collagen and gelatin were serially diluted with PBS (final volume, 0.06 ml per polystyrene tube) in a polystyrene tube (Eiken Co., Tokyo, Japan). After dilution, 0.06 ml of the solution of each fibronectin (8.5 μg/ml) was added and mixed with various diluted collagens or gelatin. Then each mixture was incubated at 37°C for 30 minutes (Fig. 3-A). At the end of that time, 100 μl of each mixture was transferred to flat-bottomed microtiter plates that had been precoated with 10 μg/ml of gelatin (Fig. 3-B). After incubation, the amount of fibronectin bound to solid-phase gelatin was quantified in exactly the same manner as the direct binding assay.

RESULTS

The proteins eluted from the column were analyzed by SDS-polyacrylamide gel electrophoresis after reduction (Fig. 4). The migrations of FN-Y and FN-O were the same.

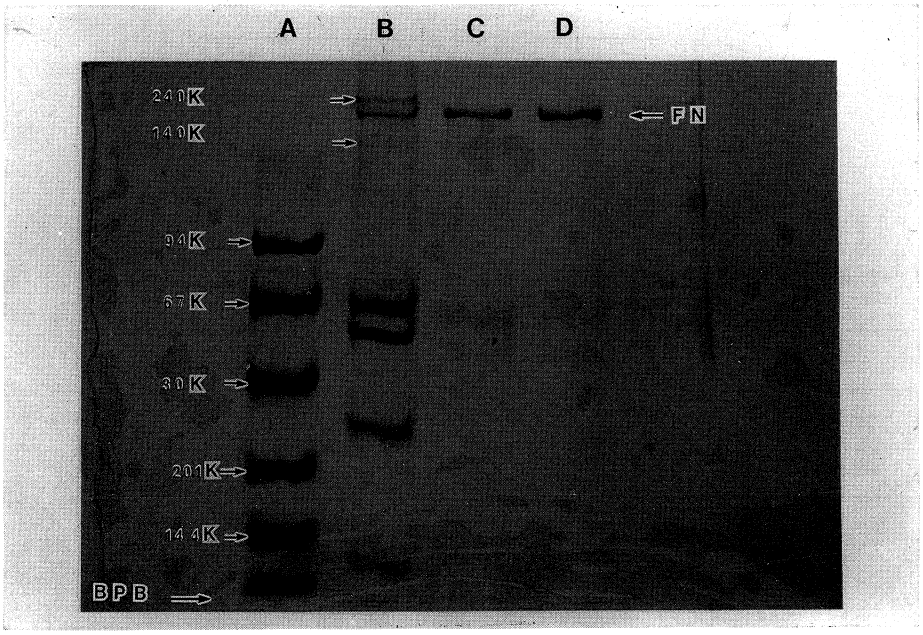

Fig. 4. SDS-polyacrylamide gel electrophoresis of human plasma fibronectin. Fibronectins were isolated by a gelatin-sepharose 4B column. Lanes A and B are molecular weight markers. Lane C shows plasma fibronectin isolated from young people (FN-Y). Lane D shows plasma fibronectin isolated from old people (FN-O).

ELISA analysis

1) Interaction of human plasma fibronectin (FN-Y, FN-O) with antihuman plasma fibronectin (1:2000 dilution) using an ELISA

FN-Y and FN-O were quantified as described earlier[3] using an ELISA. Then the concentrations of these fibronectins were equalized with 0.01 M CAPS buffer (85 μg/ml). When they were added to the wells of flat-bottomed microtiter plates that had been precoated with antihuman fibronectin IgG fraction (Cappel Lab., Westchester, NY) (1:2000) (Fig. 2), FN-Y and FN-O both bound equally well to the plastic substrate (Fig. 5, Table 1).

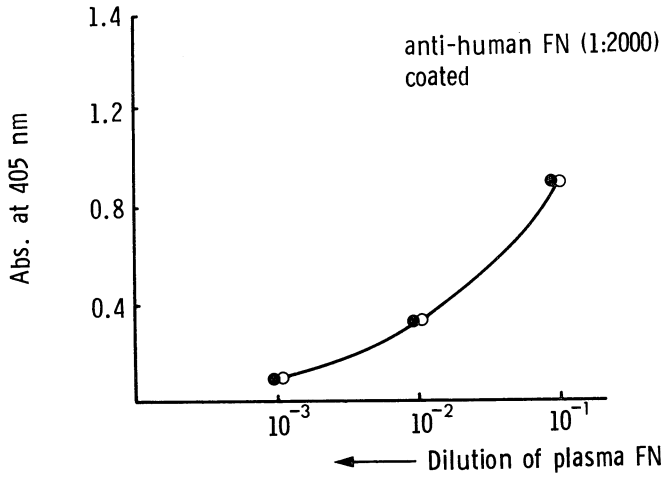

Fig. 5. Interaction of human plasma fibronetin with antihuman plasma fibronectin (1:2000) using an ELISA.
●——●; FN-Y (85 μg/ml) ○——○; FN-O (85 μg/ml)

TABLE 1. Interaction of human plasma fibronectin (FN-Y, FN-O) with antihuman plasma fibronectin (1:2000) using an ELISA.

	Dilution of plasma FN	FN-Y Mean ± S.E.(O.D)	FN-O Mean ± S.E.(O.D)	
Anti-human plasma FN adsorbed	10^{-1}	0.929 ± 0.025	0.940 ± 0.022	n.s
	10^{-2}	0.316 ± 0.011	0.337 ± 0.028	
	10^{-3}	0.109 ± 0.010	0.110 ± 0.023	

Statistical difference between FN-Y and FN-O
n.s=not significant
FN-Y=young plasma fibronectin (85 μg/ml)
FN-O=old plasma fibronectin (85 μg/ml)

2) Interaction of human plasma fibronectin with substrate adsorbed collagen using an ELISA

In a direct protein binding ELISA, gelatin, Type I collagen or Type III collagen, which was coated onto microtiter plates, was incubated with fibronectin (FN-Y, FN-O) at concentrations ranging from 0.085 to 8.5 μg/ml. Unbound

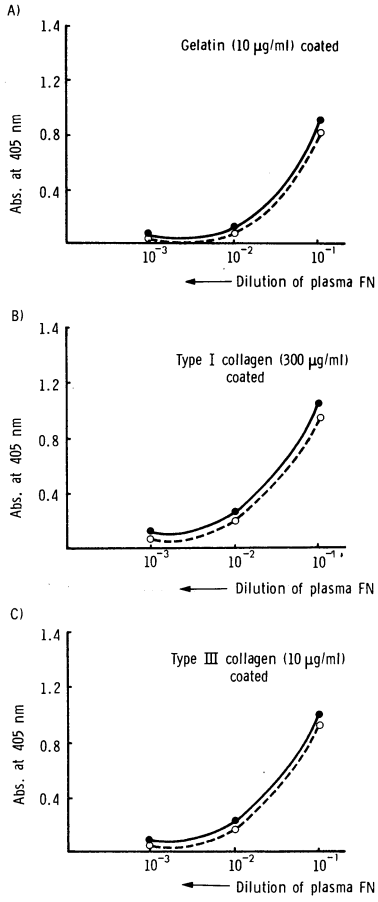

Fig. 6. Interaction of human plasma fibronectin with substrate-adsorbed collagen using an ELISA.
A) Interaction of plasma fibronectin (FN-Y, FZ-O) with gelatin.
B) Interaction of plasma fibronectin (FN-Y, FN-O) with Type I collagen.
C) Interaction of plasma fibronectin (FN-Y, FN-O) with Type III collagen.
●——●; FN-Y (85 μg/ml) ○·····○; FN-O (85 μg/ml)

fibronectin was removed, and the amount remaining bound to collagen was quantified using peroxidase-conjugated antihuman plasma fibronectin. The absorbance caused by interaction of FN-Y with collagen was compared with that of FN-O and it was found that FN-Y was more active in binding to Type I collagen, Type III collagen and gelatin than FN-O (Fig. 6, Table 2).

3) Interaction of human plasma fibronectin with solubilized collagen using a competitive inhibition ELISA

The ability of FN-Y and FN-O to bind to Type I collagen, Type III collagen and gelatin in solution was measured by an indirect protein-protein ELISA. In this assay, fibronectin (8.5 μg/ml) was first mixed with various

TABLE 2. Interaction of human plasma fibronectin with substrate-adsorbed collagen using an ELISA.

		Dilution of plasma FN	FN-Y Mean ± S.E. (O.D)	FN-O Mean ± S.E. (O.D)	
A)	Gelatin (10 μg/ml) adsorbed	10^{-1}	0.894 ± 0.016	0.813 ± 0.033	*
		10^{-2}	0.144 ± 0.022	0.106 ± 0.005	
		10^{-3}	0.070 ± 0.015	0.040 ± 0.008	
B)	Type I (300 μg/ml) collagen adsorbed	10^{-1}	1.035 ± 0.005	0.961 ± 0.014	*
		10^{-2}	0.254 ± 0.015	0.199 ± 0.007	
		10^{-3}	0.110 ± 0.015	0.088 ± 0.010	
C)	Type III (10 μg/ml) collagen adsorbed	10^{-1}	0.984 ± 0.020	0.892 ± 0.009	*
		10^{-2}	0.201 ± 0.014	0.165 ± 0.003	
		10^{-3}	0.077 ± 0.009	0.066 ± 0.008	

Statistical difference between FN-Y and FN-O
*$p<0.01$
FN-Y=young plasma fibronectin (85 μg/ml)
FN-O=old plasma fibronectin (85 μg/ml)

TABLE 3. Interaction of human plasma fibronectin with solubilized collagen using a competitive inhibition ELISA.

		Dilution of solubilized collagen (μg/ml)		FN-Y Mean ± S.E. (O.D)	FN-O Mean ± S.E. (O.D)	
A)	Gelatin (10 μg/ml) adsorbed	Gelatin	100	0.144 ± 0.013	0.207 ± 0.092	
			10	0.351 ± 0.023	0.527 ± 0.021	
			1	0.770 ± 0.056	1.110 ± 0.049	*
B)	Gelatin (10 μg/ml) adsorbed	Type I collagen	1500	0.577 ± 0.055	0.887 ± 0.063	
			150	1.093 ± 0.035	1.275 ± 0.058	**
C)	Gelatin (10 μg/ml) adsorbed	Type III collagen	500	0.060 ± 0.024	0.090 ± 0.010	
			50	0.181 ± 0.022	0.312 ± 0.063	
			10	0.649 ± 0.023	0.830 ± 0.030	*

Statistical difference between FN-Y and FN-O
*$p<0.01$ **$p<0.05$
FN-Y=young plasma fibronectin (8.5 μg/ml)
FN-O=old plasma fibronectin (8.5 μg/ml)

concentrations of Type I collagen, Type III collagen or gelatin at 37°C for 30 minutes. The amount of unbound fibronectin remaining in the solution was assayed by its subsequent reaction with substrate-adsorbed gelatin (Fig. 3). The results were confirmed by the direct binding assay. FN-O was active than FN-Y in binding to collagen and gelatin (Fig. 7, Table 3).

DISCUSSION

Age-specific defects in the biological activity of cellular fibronectin were reported by Chandrasekhar *et al.*[2,5] We supposed that plasma fibronectin like

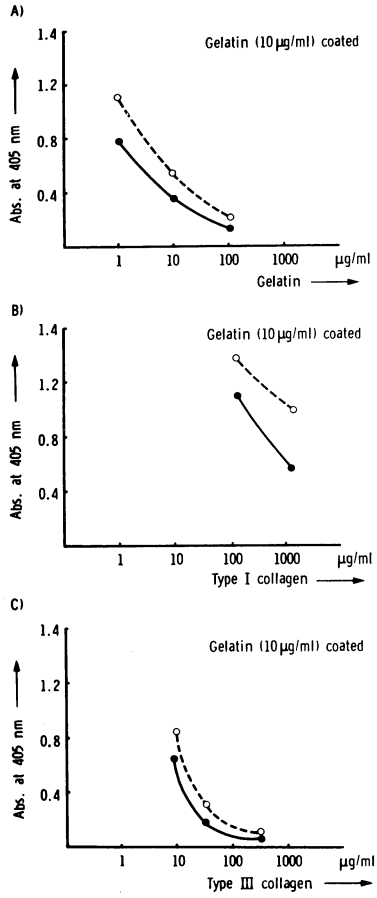

Fig. 7. Interaction of human plasma fibronectin with solubilized collagen using a competitive inhibition ELISA.
A) Interaction of plasma fibronectin (FN-Y, FN-O) with solubilized gelatin.
B) Interaction of plasma fibronectin (FN-Y, FN-O) with solubilized Type I collagen.
C) Interaction of plasma fibronectin (FN-Y, FN-O) with solubilized Type III collagen.
●——●, FN-Y (8.5 μg/ml) ○·····○; FN-O (8.5 μg/ml)

cellular fibronectin might also exhibit defects with aging. Therefore we decided to investigate changes in the quality of plasma fibronectin with aging, especially in the interaction of plasma fibronectin with collagen using an ELISA. No differences between FN-Y and FN-O were detected in molecular weights by SDS-polyacrylamide gel electrophoresis and FN-O bound poorly to gelatin, Type I and Type III collagens. Regarding cellular fibronectin, however, Chandrasekhar *et al.*[5] reported that in comparison with fibronectin isolated from early passage cells, fibronectin from late passage cells bound poorly to Type I and Type II collagens but no differences were observed in binding of the two fibronectins to gelatin. Comparison of molecular weights by SDS-polyacrylamide gel electrophoresis revealed that of fibronectin from late-passage cells was larger

than that from early-passage cells. We suppose that these differences in changes in the quality of biological activity with aging between plasma and cellular fibronectins were caused by their structural differences.[11,12] The binding differences observed between FN-Y and FN-O may be explained by structural differences in the collagen binding domain of these two fibronectins (FN-Y, FN-O) or the presence or absence of other factors that may copurify with fibronectin from plasma. Presently we are investigating why differences between FN-Y and FN-O are observed in finding with gelatin, Type I and Type III collagens.

We speculate that the changes in the quality of plasma fibronectin with aging are related to the increase in plasma fibronectin concentration with aging.[6,7]

Acknowledgment

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