Aging-associated Changes in Indices of Thrombin Generation and Protein C Activation in Humans

Normative Aging Study

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Abstract

In view of the known association of vascular disease with increasing age, we have conducted an analysis of hemostatic system activity with respect to perturbations induced by aging phenomena. We have utilized an immunochemical assay for prothrombin fragment F_{1+2} to quantify Factor Xa activity upon prothrombin in the plasma of 199 healthy males between the ages of 42 and 80. The levels of F_{1+2} in this population generally increased as a function of age ($P < 0.0001$). The metabolic behavior of this marker was determined in 10 individuals > 65 yr of age with varying levels of F_{1+2}, which ranged from 1.28 to 5.85 nM. The elevations in the concentration of this component were not due to diminished clearance of the fragment. Radioimmunoassays for fibrinopeptide A (FPA) and the protein C activation peptide (PCP) were subsequently employed to measure thrombin activity upon fibrinogen and thrombin-thrombomodulin activity upon protein C, respectively, in 82 members of this population ranging in age from 42 to 80. Significant positive correlations were again observed between increasing age and the level of F_{1+2} ($P < 0.0001$) as well as FPA ($P < 0.01$) and PCP ($P < 0.002$). The results of this cross-sectional study indicate that many apparently normal males of increasing age with normal immunologic levels of antithrombin III and protein C exhibit a biochemical defect that denotes the presence of an acquired prethrombotic state.

Introduction

Numerous investigators have attempted to determine whether a relationship exists between abnormalities in the hemostatic mechanism and the occurrence of vascular disease in populations undergoing the normal aging process. The major difficulty encountered in unravelling this puzzle has been a lack of reliable techniques for quantifying pertinent changes in blood coagulability. It should be noted that previous methods for monitoring these processes have been directed at measuring the levels of zymogens, inhibitors, or substrates of the hemostatic system (1–5). Unfortunately, these molecular species are present in large excess within the blood. Only a small percentage of the zymogens are converted to active enzymes under in vivo conditions (6–8). This level of activated intermediate is responsible for only minimal depletion of inhibitors and negligible conversion of substrates to final products. Thus, attempts to define an acquired prethrombotic state by quantifying the concentrations of zymogens, inhibitors, and substrates have yielded ambiguous results.

During the past 15 yr, a number of important aspects of the hemostatic mechanism have been elucidated. Based upon this information, a series of highly sensitive and specific RIAs have been developed which can quantify the activities of various steps of the hemostatic mechanism in vivo at the subnanomolar level. We have established assays for the F_{1+2} fragment and the protein C activation peptide (PCP)[1] that measure the cleavage of the prothrombin molecule by Factor Xa (9, 10) and the scission of protein C by the thrombin-thrombomodulin complex (8), respectively. Nossel and co-workers (11–13) have developed a similar assay for fibrinopeptide A (FPA) that monitors the cleavage of fibrinogen by thrombin. We have previously utilized the RIAs for F_{1+2} and FPA to demonstrate that substantial elevations in Factor Xa activity but not thrombin activity regularly occur in the blood of asymptomatic individuals with hereditary deficiencies of antithrombin III (7) or protein C (Bauer, K. A., A. W. Broekmans, R. M. Bertina, J. Conard, M. Horellou, M. M. Samama, and R. D. Rosenberg. Hemostatic enzyme generation in the blood of patients with hereditary protein C deficiency. Submitted for publication), disorders known to be correlated with the subsequent development of thrombosis.

In this communication, we have utilized the preceding immunochemical techniques to demonstrate that many healthy individuals exhibit excessive activity of their hemostatic mechanisms in association with the aging process in the absence of clinically overt vascular disease.

Methods

Patient selection. Participants of the Normative Aging Study of the Veterans Outpatient Clinic, Boston, MA, were studied during a 60-mo period from January 1981 to December 1985. The Normative Aging Study is a longitudinal and interdisciplinary investigation of aging phenomena in males (14). The population panel was established in 1963 by screening 6,000 males for possible entry into the study with the subsequent selection of 2,280 healthy individuals from this cohort. The ages of the subjects ranged from 21 to 81. The excluded group was identified by history and physical examination as well as laboratory investigation. Primary causes for disqualification were chronic disorders such as heart disease, cancer, peptic ulcer, gout, bronchitis, sinusitis, asthma, hypertension, glucose intolerance, cataracts, chronic internal eye disease, and impaired hearing.

Since 1963, Normative Aging Study subjects have begun to differentiate into minor subpopulations with specific diseases, as well as a

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Received for publication 15 January 1987 and in revised form 8 July 1987.

The Journal of Clinical Investigation, Inc.
Volume 80, December 1987, 1527–1534

1. *Abbreviations used in this paper:* FPA, fibrinopeptide A; PCP, protein C activation peptide.

major subpopulation that has remained healthy. The social characteristics of this population reveal that they come from a slightly higher socioeconomic level than the general population. In addition, Blacks are somewhat underrepresented within the sample. The population sample was initially chosen to obtain a geographically stable group and < 1% of patients per year have become unavailable for further investigation due to all causes, including death.

Individuals in the Normative Aging Study are examined once every 5 yr up to the age of 52 and once every 3 yr thereafter. This procedure includes a detailed history, a complete physical examination, chest x ray, pulmonary function tests, and electrocardiogram, as well as standard blood and urine tests. The latter analyses include complete blood count, liver and kidney function tests, glucose tolerance test, lipid profile, and protein electrophoresis. Diseases uncovered during the examination as well as those occurring between visits are recorded by standardized techniques. Furthermore, psychosocial data of various types are prospectively collected during the Normative Aging Study. These include the incidence and intensity of smoking as well as the level of physical activity of the participants.

Collection of blood samples. Venipunctures were performed atraumatically in the morning on fasting subjects with 19- or 21-gauge butterfly infusion sets using a two-syringe technique. Patients were instructed not to take aspirin or aspirin-containing compounds for a minimum of 1 wk before sample collection. Blood samples were drawn into plastic syringes preloaded with the appropriate solutions, including the following. (*a*) For immunologic measurements of antithrombin III, protein C, and prothrombin, 3.8% (wt/vol) sodium citrate was employed; the ratio of anticoagulant to blood used was 0.1:0.9 (vol/vol). (*b*) "F_{1+2} anticoagulant": 38 mM citric acid, 75 mM sodium citrate, 136 mM dextrose, 6 mM EDTA, 6 mM adenosine, and 25 U/ml heparin. The ratio of anticoagulant to blood employed was 0.2:1.0 (vol/vol). (*c*) "FPA anticoagulant": This preparation was provided by Mallinckrodt, Inc., St. Louis, MO. The ratio of anticoagulant to blood used was 0.1:0.9 (vol/vol). After collection of blood samples, plasma fractions were obtained by centrifugation at 4°C for 15 min at 1,600 *g* and stored at −80°C before use.

A normal plasma pool was constructed by pooling equal volumes of plasma from 30 control subjects. This population consisted of healthy laboratory and medical personnel between the ages of 20 and 40 who gave a negative history for bleeding as well as thrombosis, and were not taking any medications at the time of sample collection.

Processing of plasma for PCP RIA. The PCP in 6–12 ml of plasma was extracted from other plasma constituents and concentrated ~ 10-fold as outlined previously (8). The larger proteins were initially precipitated by adding 0.1 vol of 7 M perchloric acid to 1 vol of plasma. After 15 min of incubation in an ice bath, the samples were centrifuged at 27,000 g for 20 min at 4°. To salt out the residual perchlorate ions, we poured the supernatant fluid into a centrifuge tube and a sufficient amount of a solution of 3.5 M potassium hydroxide was added to raise the pH to > 7. After a second centrifugation, the supernatant was acidified with an amount of 10% (vol/vol) phosphoric acid sufficient to lower the pH of the solution to < 3. A Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) was then washed with 5 ml of 80% (vol/vol) methanol followed by 5 ml of 0.1% (vol/vol) phosphoric acid. The sample was applied to the cartridge and the matrix was washed with 4 ml of distilled water. The peptide was subsequently eluted with 4 ml of 80% (vol/vol) methanol into 12 × 75-mm test tubes. The contents of the tubes were evaporated to dryness overnight in a Savant Speed Vac Concentrator. Samples were individually reconstituted with 1.2 ml of 0.10 M NaCl in 0.05 M Tris-HCl, pH 7.5, containing 0.02% (wt/vol) sodium azide and 1 mg/ml ovalbumin. The specimens were then assayed by RIA for PCP immunoreactivity.

Immunoassays. The plasma concentrations of antithrombin III, prothrombin, protein C, and F_{1+2} were determind by double-antibody RIA as described in earlier reports from our laboratory (8, 10, 15). The plasma levels of FPA were established by RIA utilizing a kit provided by Mallinckrodt, Inc.

The PCP RIA was also performed by using a double-antibody approach. Radiolabeling of tyrosinated PCP was carried out by the chloramine T method of Greenwood et al. (16) using 1 μg of peptide and 1 mCi of carrier-free $Na^{125}I$ (New England Nuclear, Boston, MA). After separation of the peptide from free iodide by Sephadex G-10 gel filtration, > 70% of the labeled material could be bound by high concentrations of antisera directed against this component. Antisera to PCP was raised in rabbits as outlined previously (8). The initial assay reaction mixtures were composed of 50 μl of radiolabeled tyrosinated PCP (~ 10,000 cpm), 500 μl of unlabeled peptide standards or unknown sample, as well as 100 μl of the PCP antibody population. All of the reagents had been extensively diluted in Tris-buffered saline (0.05 M Tris-HCl, 0.10 M NaCl, 0.02% (wt/vol) sodium azide, pH 7.5, containing 1 mg/ml ovalbumin). The sensitivity of the assay was maximized by diluting the crude antiserum 1,500-fold. Under these conditions, ~ 33% of the ^{125}I-labeled counts were immunoprecipitable. To act as a carrier in the second antibody separation, 5% (vol/vol) nonimmune rabbit serum was added to the solution containing the tracer. The tubes were mixed and then incubated at 4°C for 18 h. Thereafter, radiolabeled antigen bound to the antibody was separated from ^{125}I-labeled tyrosinated peptide. This was accomplished by adding 1 ml of 1% (vol/vol) goat anti-rabbit IgG, 3.4% (wt/vol) polyethylene glycol (PEG) 6000 in Tris-buffered saline to each tube. The relative amounts of nonimmune rabbit sera as well as goat anti-rabbit IgG were chosen to give the maximal precipitation of radiolabeled antigen. The tubes were centrifuged at 4°C for 20 min at 1,800 g and washed once at 4°C with Tris-buffered saline. The resultant precipitates were quantified for ^{125}I counts.

In the PCP RIA, we have observed that the average recovery of added dodecapeptide in the extraction procedure is extremely constant at ~ 60% and have elected to not divide the values obtained by the fractional recovery. Therefore, the levels of PCP cited for normal individuals are lower than previously reported (8).

^{131}I-F_{1+2} preparation and turnover studies. Human F_{1+2} was purified from 2 liters of fresh frozen plasma obtained from four healthy donors who had been carefully screened for hepatitis and acquired immune deficiency syndrome. The plasma was allowed to warm to 24°C and clotting was initiated by the addition of $CaCl_2$ (final concentration, 32.4 mM). After 90 min of incubation, the clot was removed, heparin (final concentration, 1 U/ml) was added, and the suspension was stirred for an additional 5 min. Subsequently, F_{1+2} was isolated from the sera by DEAE-cellulose batch adsorption-elution and hydroxylapatite chromatography as described by Aronson et al. (17). Examination of a series of fractions eluted from hydroxylapatite with a potassium phosphate concentration of 0.35–0.45 M, revealed that > 90% of the resultant material was F_{1+2} as judged by the SDS gel electrophoretic technique of Laemmli (18). To remove minor higher and lower molecular weight components that migrated at rates similar to those of prothrombin and F_1, respectively, 2 mg of F_{1+2} in 15 ml of 0.02 M Tris-HCl, 0.05 M NaCl, pH 7.5, were filtered at 20 ml/h through a column of heparin-Sepharose (0.8 × 13 cm) equilibrated with the same buffer. The heparin-Sepharose was sterilized during its preparation, and had not previously been used for any other purpose. The matrix was then washed with the above buffer. This procedure was conducted at 4°C. The recovery of F_{1+2} by absorbance measurements averaged 90% of the total amount applied to the column. Analysis of the resultant product by SDS gel electrophoresis confirmed that the minor contaminating species had been eliminated. The overall yield from 2 liters of plasma averaged ~ 3.6 mg. All buffers were made in sterile, pyrogen-free water, and all glassware had been autoclaved before use.

Radiolabeling of F_{1+2} was accomplished by the method of Greenwood et al. (16) utilizing 15 μg of F_{1+2} and 0.6 mCi of carrier free $Na^{131}I$ (New England Nuclear). Upon completion of the iodination, human serum albumin (Cutter Biological, Berkeley, CA) was added to the reaction mixture (final concentration, 10 mg/ml). Separation of free iodide from labeled protein was achieved by filtration of samples at 15

ml/h through a sterile column (0.8 × 26 cm) of Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.1 M potassium phosphate, pH 7.0. Fractions of 0.5 ml were collected in sterile plastic tubes with screw caps, and those within the first peak of radioactivity were examined with respect to their ability to be precipitated with 10% (wt/vol) TCA and specific F_{1+2} antisera. Fractions were pooled in which the labeled material was > 95% precipitable with TCA and > 80% immunoprecipitable by specific F_{1+2} antisera. This material was frozen at −80°C and utilized within 4 d of preparation. Before intravenous injection, the iodinated F_{1+2} preparation was brought to a final volume of 3–5 ml in sterile 0.9% (wt/vol) NaCl containing human serum albumin (final concentration, 2 mg/ml) and passed through a 0.22-μm filter (Millipore Corp., Bedford, MA). The labeled material was demonstrated to be sterile by standard microbiologic techniques, and pyrogen-free by the Limulus test (19).

Each subject was given Lugol's solution (three drops twice daily) on the day before injection of ^{131}I-F_{1+2} and for the next 10 d to prevent uptake of the radioactive iodine by the thyroid. ~ 40 μCi of ^{131}I-F_{1+2} were infused as a bolus into a peripheral arm vein of each volunteer. A 19- or 21-gauge butterfly needle was inserted into a vein on the opposite arm and serial samples of 5 ml of blood were drawn by two-syringe technique into "F_{1+2} anticoagulant." During intervals between specimen collection, a solution of 5% (wt/vol) dextrose was administered to the subjects through this line at a rate of ~ 50 ml/h. The blood samples were processed and 0.5-ml aliquots of plasma were counted in a Gamma 8000 Counting System (Beckman Instruments, Inc., Irvine, CA).

Informed consent. All clinical studies and informed consent procedures were approved by the Committee on Clinical Investigations, New Procedures, and New Forms of Therapy of the Beth Israel Hospital and the Human Studies Committee of the Veterans Administration Outpatient Clinic. The procedures utilized in carrying out the ^{131}I-F_{1+2} turnover studies were also accepted by the hospital's Radioactive Drug Research Committee.

Analysis of data. Estimation of relative immunoreactivity, computation of the slopes of the dose-response curves, as well as determinations of the various associated indices were obtained by a least-squares fit of the RIA results to a "four parameter" model as described by Rodbard (20, 21). Statistical analyses of data were conducted by standard techniques (22). In most instances, the means are provided with associated standard deviations.

Results

We have measured the levels of prothrombin activation fragment F_{1+2} in the plasma of 199 healthy male participants of the Normative Aging Study between the ages of 42 and 80. This population was constructed by carefully screening the medical records of Normative Aging Study participants to exclude those individuals who had developed coexistent medical conditions (i.e., venous or arterial thrombotic disease, heart disease, peripheral vascular disease, hypertension, diabetes, cancer, renal disease, liver dysfunction, etc.) that might cause alterations in hemostatic system activity as measured by our RIAs. A scattergram with associated mean values for seven different age groups is presented in Fig. 1. The mean F_{1+2} levels in the groups with ages 40–44, 45–49, 50–54, 55–59, 60–64, 65–69, and 70–80 were 1.33 nM±0.44 (n = 8), 1.77 nM±0.74 (n = 44), 1.90 nM±0.77 (n = 35), 1.76 nM±0.59 (n = 49), 2.19 nM±0.99 (n = 26), 2.09 nM±0.68 (n = 9), and 3.08 nM±1.55 (n = 28), respectively. The data indicate that there is a general increase in the average level of F_{1+2} as a function of age. This appears to be due to a growing number of individuals within each advancing age group who exhibit markedly elevated

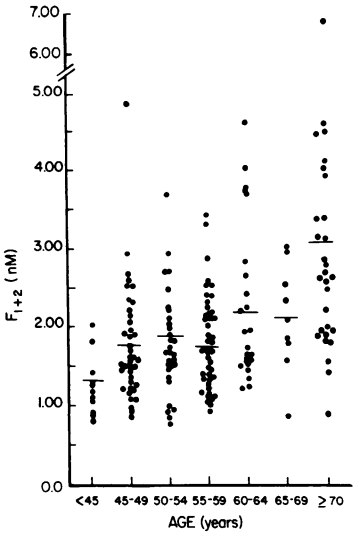

Figure 1. Plasma F_{1+2} levels in seven different age groups of Normative Aging Study participants. Horizontal bars represent the mean value of the parameter in each patient group. When concentrations of F_{1+2} were determined in a given individual at multiple time points, the value represents the determination at the initial sampling.

values of this parameter. Statistical analysis reveals that plasma levels of F_{1+2} correlate strongly with age (r = 0.417, P < 0.0001).

It should be noted that this cohort of subjects included 22 smokers ranging in age from 45 to 77. The mean level of F_{1+2} in this group was 2.11 nM±0.62. Due to the small number of such individuals in our study population, it is not possible to draw any conclusions regarding the influence of this variable on hemostatic system activity. However, we have reanalyzed our data after excluding these subjects and the statistical parameters obtained on correlating plasma F_{1+2} levels with age give a positive result similar to that cited above.

These results suggest that significant elevations in the levels of F_{1+2} occur regularly in healthy males undergoing the aging process. To demonstrate that the level of F_{1+2} represents a stable parameter that is characteristic of a particular individual at a given point in time, we have obtained blood samples from 14 individuals on more than one occasion at intervals ranging from months to several years (Table I). The values were statistically indistinguishable from those obtained on the initial specimens (P > 0.20). The plasma F_{1+2} concentration in subjects 4 and 8 however decreased approximately twofold.

To demonstrate that increased values of F_{1+2} within aged subjects are due to excessive production rather than reduced clearance of this component, the metabolic behavior of this marker was determined in 10 healthy Normative Aging Study participants > 65 yr of age (mean age, 73.7±4.2) with levels of F_{1+2} ranging from 1.28 to 5.85 nM (Table II). Plasma radioactivity measurements in these individuals were plotted against time; ^{131}I-F_{1+2} plasma radioactivity is described by a two-exponential curve, $C_1e^{-r_1t} + C_2e^{-r_2t}$ (23). The fractional breakdown rate, k_B (h^{-1}), was calculated from $[C_1/r_1 + C_2/r_2]^{-1}$ (24). Whereas some differences in fractional breakdown rates were observed between the various individuals, a significant relationship between this parameter and the plasma level of F_{1+2} was not observed (P > 0.10). Therefore, the elevations in the concentration of this marker in these people were not due to diminished clearance of the fragment. We have observed however that the fractional breakdown rate, k_B, in 10 subjects under age 50 (mean age, 32.6±11.3) is 0.310 h^{-1}±0.06. While this value is somewhat greater than that observed in the sub-

Table I. Normative Aging Study Patients Studied on Multiple Occasions

Subject	Age	F_{1+2}	FPA	PCP
		nM	*nM*	*pM*
1	52	1.37	—	—
	55	1.19	1.11	1.58
2	51	1.45	—	—
	54	1.60	0.483	1.20
3	53	0.963	—	—
	56	1.12	0.613	1.93
4	51	3.67	—	—
	55	1.81	1.63	1.98
5	42	1.30	—	—
	44	1.80	0.956	2.48
6	67	1.79	—	—
	70	1.41	1.01	2.35
	71	1.28	0.979	1.76
7	72	3.33	1.71	1.64
	72	3.08	2.39	2.08
8	74	3.11	1.36	2.26
	74	1.77	1.15	2.14
9	74	2.80	1.06	2.62
	74	3.14	1.11	2.52
10	72	1.89	0.501	2.20
	72	2.46	0.951	2.25
11	72	4.55	0.994	2.82
	73	4.36	1.05	2.80
12	80	3.40	1.67	2.50
	80	3.16	1.38	1.80
13	76	2.01	1.59	4.15
	76	2.04	1.12	3.70
14	72	1.78	0.65	1.65
	72	1.58	1.22	1.31

jects over age 65 ($P < 0.05$), it is apparent that this difference in clearance rates cannot make a significant contribution to the greater than twofold elevations in F_{1+2} levels seen in many aged subjects.

The preceding data indicate that many apparently healthy individuals undergoing the normal aging process have excessive plasma Factor Xa activity as measured by the F_{1+2} assay. We next undertook more detailed studies of hemostatic system activity in Normative Aging Study participants in an attempt to determine the nature of the defect leading to elevated F_{1+2} levels. The study population consisted of 82 healthy males derived from the original population of 199 subjects who consented to visiting our research laboratory for further investigation. The plasma levels of F_{1+2}, FPA, and PCP were quantified by RIA and plotted as a function of age (Fig. 2). Measurements of antithrombin III, protein C, and prothrombin antigen were also performed (Table III). Significant positive correlations were again noted between increasing age and the level of F_{1+2} ($r = 0.461$; $P < 0.0001$) as well as FPA ($r = 0.296$; $P < 0.01$) and PCP ($r = 0.346$; $P < 0.002$). The levels of antithrombin III, protein C, and prothrombin antigen did not display statistically significant changes with increasing age.

It should be noted that five patients with varying ages had FPA values over 3.5 nM (Fig. 2 *B*). The mean F_{1+2} and PCP levels in this cohort were 2.11 nM±0.64 and 1.66 pM±0.78, respectively. Given the lack of concordance of these measurements, it is possible that the elevated FPA measurements in these individuals were produced during the venipuncture procedure. We have therefore reanalyzed our data after excluding these five people and a highly significant correlation was again noted between FPA and age ($r = 0.395$; $P < 0.0005$).

We have obtained additional specimens for assay from nine Normative Aging Study participants within a half year of the initial sampling to demonstrate that a given plasma level of FPA or PCP represents a stable parameter that is characteristic of a particular individual at a given point in time (Table I). As was the case for F_{1+2} levels, the levels of these species were statistically indistinguishable from those obtained initially (for FPA, $P > 0.05$; for PCP, $P > 0.10$).

To examine the interrelationships among F_{1+2}, FPA, PCP, antithrombin III, protein C, and prothrombin, we correlated the simultaneous levels of the various species in the blood of these 82 individuals. There were no statistically significant correlations observed between the levels of F_{1+2} and FPA, PCP, antithrombin III, protein C, or prothrombin. A signifi-

Table II. Parameters from Turnover Studies Using ^{131}I-F_{1+2} in Normative Aging Study Subjects

Subject	Age	F_{1+2}*	C_1	r_1	C_2	r_2	k_B
		nM					
6	71	1.49	0.765	0.225	0.198	6.80	0.292
7	72	3.21	0.772	0.177	0.225	2.28	0.224
8	74	2.44	0.676	0.259	0.278	4.62	0.374
9	74	2.97	0.763	0.186	0.225	3.36	0.240
12	80	3.28	0.796	0.196	0.208	2.02	0.241
13	76	2.03	0.708	0.201	0.288	4.20	0.279
14	72	1.68	0.534	0.121	0.481	0.423	0.180
15	72	1.28	0.775	0.230	0.238	3.21	0.291
16	80	5.85	0.877	0.165	0.123	1.99	0.186
17	66	3.60	0.776	0.181	0.217	3.90	0.230

^{131}I-F_{1+2} plasma radioactivity is described by a two-exponential curve, $C_1e^{-r1t} + C_2e^{-r2t}$ (23). The fractional breakdown rate, k_B (h^{-1}), was calculated from $(C_1/r_1 + C_2/r_2)^{-1}$ (24). *Plasma F_{1+2} levels cited represent mean of determinations obtained at different time points.

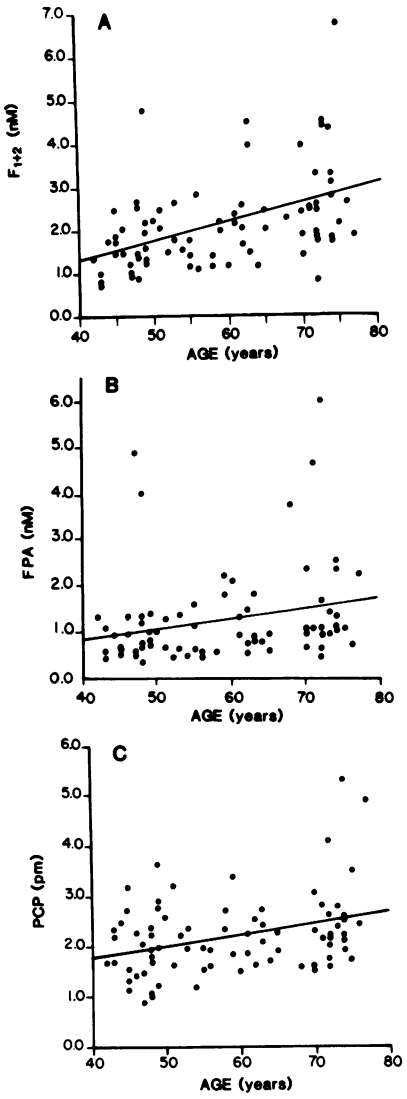

Figure 2. Concentration of F_{1+2} (*A*), FPA (*B*), and PCP (*C*) as a function of age in 82 Normative Aging Study participants. Lines represent the best linear squares fit of the data.

cant correlation was observed between the concentrations of prothrombin and protein C ($r = 0.645$; $P < 0.0001$).

Our results demonstrate that the levels of F_{1+2}, FPA, and PCP increase with advancing age. The measurements of FPA and PCP reflect the in vivo enzymatic activity of thrombin upon fibrinogen and the amount of activated protein C generated by thrombin bound to thrombomodulin, respectively. A significant correlation between the levels of the three markers of hemostatic system activation however was not observed. To ascertain whether we might be able to identify subsets of aging patients with a common molecular basis for their observed hemostatic defect, we have chosen to correlate the levels of the above parameters in a subset of 53 individuals who are generating high and low levels of thrombin within their circulatory systems. The groups consisted of 22 males with $F_{1+2} \geq 2.5$ nM (mean, 3.47 nM±1.09; mean age, 65; range, 45–80 yr) and 31 males with $F_{1+2} \leq 1.7$ nM (mean, 1.29 nM±0.25; mean age, 52; range, 43–72 yr). Mean levels of FPA and PCP in these two groups of patients were 1.46 nM±1.09 and 2.35 pM±0.90, and 1.01 nM±0.85 and 1.89 pM±0.49, respectively. Analysis of the data for F_{1+2} vs. FPA or PCP revealed a significant correlation coefficient for PCP only ($r = 0.417$; $P < 0.002$).

We have also compared subsets of patients who have high and low plasma levels of FPA. The groups consisted of 17 males with FPA ≥ 1.5 nM (mean, 2.80 nM±1.36; mean age, 65) and 23 males with FPA ≤ 0.7 nM (mean, 0.576 nM±0.09; mean age, 52). A similar comparison was carried out for 21 subjects with PCP ≥ 2.5 pM (mean, 3.21 pM±0.77; mean age, 62) and 23 subjects with PCP ≤ 1.7 pM (mean, 1.46 pM±0.24; mean age, 54). Analysis of the data for FPA vs. F_{1+2} or PCP and PCP vs. F_{1+2} or FPA did not give any statistically significant correlations.

Discussion

A number of highly sensitive and specific immunologic assays have been developed to quantify the activity of various steps of the hemostatic mechanism in vivo (8–13). The study of aging-related changes in blood coagulation however has been limited to the monitoring of alterations in the levels of platelet release proteins such as beta-thromboglobulin and platelet factor 4 (25–28). Zahavi et al. (29) have observed significant elevations in the levels of these two alpha-granule proteins with increasing age. This age-dependent difference was most apparent in elderly females. Two other groups of investigators (30, 31) have obtained similar results in smaller numbers of patients. It is unclear, however, whether the correlations observed are due to increased activity of the coagulation mechanism, increased destruction of platelets, or decreased catabolic rates of the platelet release proteins.

We have previously evaluated the utility of immunochemical techniques which measure the extent of prothrombin activation and thrombin generation in vivo as markers of the prethrombotic state. The populations chosen for study were asymptomatic patients with inherited deficiencies of either

Table III. Levels of F_{1+2}, FPA, PCP, Antithrombin III, Protein C, and Prothrombin in 82 Normative Aging Study Participants

Age range	No. of subjects	F_{1+2}	FPA	PCP	Antithrombin III	Protein C	Prothrombin
		nM	*nM*	*pM*	*% of normal*	*% of normal*	*% of normal*
<50	28	1.68±0.81	1.10±1.00	1.97±0.70	99.5±13.6	104±17.4	114±9.8
50–69	29	2.07±0.79	1.15±0.73	2.12±0.51	92.3±13.9	103±14.8	105±15.5
≥70	25	2.78±1.26*	1.64±1.27	2.63±0.97‡	94.4±15.6	95.9±18.7	104±21.3

Data are mean±SD. *F_{1+2} levels were significantly elevated in subjects ≥ 70 yr of age as compared with those in groups < 50 yr ($P < 0.001$) or 50–69 yr ($P < 0.02$). ‡PCP levels were significantly elevated in subjects ≥ 70 yr as compared with those in groups < 50 yr ($P < 0.01$) or 50–69 yr ($P < 0.05$).

antithrombin III (7) or protein C (Bauer, K. A., A. W. Broekmans, R. M. Bertina, J. Conard, M. Horellou, M. M. Samama, and R. D. Rosenberg. Hemostatic enzyme generation in the blood of patients with hereditary protein C deficiency. Submitted for publication), disorders known to be correlated with the subsequent development of thrombosis. These two congenital disorders constitute relatively homogenous disease models for testing the proposition that an imbalance between procoagulant and anticoagulant forces must exist for a prolonged period of time before the eventual development of thrombotic phenomena. We used RIAs for F_{1+2} and FPA to quantify the levels of free Factor Xa and thrombin, respectively, within the blood of asymptomatic individuals not on anticoagulants from eight separate kindreds with congenital antithrombin III deficiency (7). The median age of this cohort was 22. This study demonstrated that plasma F_{1+2} levels were significantly elevated by about twofold in almost all patients with this disorder, whereas the concentrations of FPA were not substantially altered in these individuals as compared with an age-matched control group. The metabolic behavior of ^{131}I-F_{1+2} was found to be identical in antithrombin III–deficient patients and normal individuals. These findings suggest that patients with this inherited thrombotic disorder exhibit increased concentrations of Factor Xa which lead to excessive activation of prothrombin, but that the resulting thrombin is neutralized before it can cleave fibrinogen to any significant extent. The hemostatic system hyperactivity as measured by the F_{1+2} assay could be specifically corrected by raising the plasma antithrombin III levels of several of the individuals into the normal range by infusing purified concentrates of the inhibitor.

Marcum et al. (32–35) have provided biochemical, cell biologic as well as physiologic evidence that heparinlike proteoglycans intimately associated with the vascular endothelium can accelerate hemostatic enzyme–antithrombin III complex formation via a process that is identical to that of commercial heparin. In addition, Carlson and co-workers (36) have noted that metabolic turnover studies in humans with radiolabeled antithrombin III are consistent with a model in which ~ 10% of the total pool of antithrombin III is associated with a noncirculating vascular compartment that probably represents the heparinlike proteoglycans of the vessel wall. It therefore appears likely that a small fraction of plasma antithrombin III is normally bound to the unique heparinlike proteoglycans of the vessel wall in an activated conformation. This pool of protein probably represents the physiologically relevant protease inhibitor population, whereas the same component is of minimal functional relevance when free within the blood. Therefore, we surmise that antithrombin III–deficient patients have an insufficient plasma level of protease inhibitor to saturate the heparinlike proteoglycans of the vessel wall and hence cannot generate sufficient activated protein to suppress Factor Xa formed within the circulatory system.

We have also employed the specific RIAs for F_{1+2}, FPA, and PCP to quantify the production and action of thrombin as well as the activation of protein C within the blood of 23 asymptomatic nonanticoagulated heterozygotes from 13 kindreds with congenital protein C deficiency (Bauer, K. A., A. W. Broekmans, R. M. Bertina, J. Conard, M. Horellou, M. M. Samama, and R. D. Rosenberg. Hemostatic enzyme generation in the blood of patients with hereditary protein C deficiency. Submitted for publication). The median age of this cohort was 37. Significant increments in Factor Xa activity but not thrombin activity also were observed to regularly occur in the blood of these subjects as compared with an age-matched control group. These patients were also noted to have significantly reduced plasma levels of PCP. Based upon the above studies, it would appear that malfunction of the endogenous heparin–antithrombin III and/or protein C–thrombomodulin mechanisms will result in excessive generation of thrombin well before the development of overt thrombotic disease.

In view of the known association of vascular disease with increasing age, we have conducted a detailed analysis of hemostatic system activity with respect to perturbations induced by aging phenomena. None of these individuals exhibited an immunochemical deficiency of either antithrombin III or protein C. Our investigation reveals a highly significant positive correlation between increasing age and the plasma levels of F_{1+2}. Metabolic turnover studies of ^{131}I-labeled F_{1+2} in these subjects indicate that elevations in concentration of the fragment were not due to diminished clearance of this component.

We also observed statistically significant elevations in the levels of FPA and PCP with increasing age. However, we were unable to establish direct correlations between these parameters and the F_{1+2} measurements. It should be noted that the magnitude of the FPA and PCP elevations between older and younger subjects was considerably smaller than was the case for F_{1+2} values. This may result in part from the relatively prolonged survival of F_{1+2} in the human circulation as compared with FPA (12) or PCP (8). However, it is likely that a more rapid in vivo neutralization rate for thrombin vis a vis Factor Xa also contributes to the disparate increase in the levels of F_{1+2} as compared with FPA or PCP (7).

To identify subsets of aging patients with a common molecular basis for hemostatic system hyperactivity, we have analyzed the results in Normative Aging Study participants with high and low values in the F_{1+2}, FPA, and PCP RIAs. In a subgroup of 53 patients with F_{1+2} measurements that were either > 2.5 nM (mean F_{1+2}, 3.47 nM; mean age, 65) or < 1.7 nM (mean F_{1+2}, 1.29 nM; mean age, 52), a significant positive correlation emerged between between the levels of F_{1+2} and PCP.

Based upon the above cross-sectional analysis of a cohort population, our results indicate that many apparently normal males of increasing age with normal immunologic levels of antithrombin III and protein C exhibit a biochemical defect that denotes the presence of an acquired prethrombotic state. This pathologic situation can be biochemically defined as elevated blood levels of Factor Xa (high plasma concentrations of F_{1+2} in association with normal metabolic behavior of this marker) with normal or increased blood levels of thrombin (normal or increased plasma concentrations of FPA and/or PCP). On one hand, this situation is analogous to our findings in asymptomatic patients with congenital antithrombin III deficiency, where we have noted normal to slightly elevated FPA and PCP values in subjects with high F_{1+2} levels (unpublished data). On the other hand, this situation is in sharp contradistinction to our observations in asymptomatic individuals with congenital protein C deficiency and high F_{1+2} levels who had reduced measurements of PCP (Bauer, K. A., A. W. Broekmans, R. M. Bertina, J. Conard, M. Horellou, M. M. Samama, and R. D. Rosenberg. Hemostatic enzyme generation in the

blood of patients with hereditary protein C deficiency. Submitted for publication). The data suggest that reduced activity of the thrombomodulin receptor mechanism is not a likely etiology for the prethrombotic state associated with aging.

It therefore appears that a cohort of older males exhibits hemostatic system hyperactivity either on the basis of suppression of the endogenous heparin–antithrombin III mechanism without reduction in plasma concentration of the protease inhibitor, or excessive generation of Factor Xa that cannot be contained by this mucopolysaccharide-dependent natural anticoagulant mechanism. The decreased activity of the heparin–antithrombin III mechanism could be secondary to low levels of vessel wall heparinlike substances and/or more effective competition between blood components and antithrombin III for the limited quantities of endothelial cell glycosaminoglycan. In this regard, it is interesting to note that the incidence of thromboembolic disease in patients with congenital deficiencies of antithrombin III or protein C increases substantially with advancing age (37, 38). A further reduction in the functional activity of these anticoagulantly active heparinlike molecules with the normal aging process might explain this clinical observation. The increased generation of Factor Xa in our population of aging males could also be due to greater exposure of tissue factor with subsequent activation of Factor VII as a consequence of the presence of occult vascular disease.

The elucidation of the molecular basis for the emergence of an age-dependent hypercoagulable state will require additional research. Recent studies have provided evidence that cultured fibroblasts synthesize heparinlike molecules that exhibit anticoagulant activity (39). This system may provide a useful model for studying defects of this component of the vascular endothelium in patients undergoing the aging process. Biochemical markers such as the F_{1+2} assay which are able to reflect dysfunction of these natural anticoagulant mechanisms might then be used to develop therapeutic strategies to correct these derangements and hopefully prevent the onset of vascular disease.

Acknowledgments

This work was supported in part by National Institutes of Health grants P01 AG-00599, P01 HL-33014, and RR-01032 to the General Clinical Research Center of Beth Israel Hospital, and the Medical Research Service of the Veterans Administration.

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