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THE ROLE OF FIBRINOGEN IN CORONARY HEART DISEASE AND ITS POSSIBLE DEPENDENCE ON ACTIVATED POLYMORPHONUCLEAR LEUKOCYTES

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ABSTRACT

Fibrinogen (FB), an acute phase protein, is recognized as an independent risk factor in the plasma of patients wits coronary heart disease (CHD). The plasma FB level is also significantly increeased in inflammation and neoplasm. We performed an in vitro study showing that the chemiluminescence (CL) emission produced by zymosan-activated polymorpho-nuclear leukocites (PMNL) was directly related to the concentration of FB, fibrinopeptide A or FB-degradation products(FDP). Fibrin inhibited CL emission. We also found that the in vivo plasma levels of FB or FDP were significantly higher in the plasma of 100 cardiac catheterized patiens with CHD than in the plasma of the age and sex matched controls. In 85% of the patiens with CHD, both the plasma FB and the CL emission were significantly increased. In the other 15%, the plasma FDP level was higher than the FB level, however, the CL level was still higher than in the age and sex matched controls. Activated PMNL may increase the plasma level of FDP as a consequence of enhanced enzyme-dependent degradation of FB. Our results indicate than the role of FB and independent risk factor in CHD depends of the level of activated polymorphonuclear leukocytes in the plasma.

RESUMO

Estudos in vitro demonstraram que a emissão quimiluminescente (CL) produzida por leucócitos plimorfonucleares (PMNL) activados por zimosano está diretamente relacionada com a concentração de fibrinogênio (FB), fibrinopeptídeo A e produtos de degradação de fibrinogênio (FDP). Foi demonstrado também que os teores de FB e FDP no plasma in vivo foram consideravelmente mais elevados em pacientes cardíacos cateterizados, comparado com controles. Os leucócitos polimorfonucleares ativados podem aumentar o nível de FDP. Os resultados indicam que o papel do FB e o fator de risco independente em pacientes cardíacos cateterizados depende do nível de PMNL no plasma.

KEYWORDS: fibrinogen, chemiluminescence, polymotphonuclear leukocytes, coronary heart disease, reactive oxygen species

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INTRODUCTION

As for other acute phase proteins, such as ceruloplasmin and protein C, the plasma level of fibrinogen (FB) is significantly increased, nonspecifically in inflammations and neoplasm [1]. An increase plasma level of FB has been recognized in many studies as one of the main risk factors or biological markers in CHD [1-5]. A study of 15,000 healthy subjects [5] indicated the plasma FB level influenced the development of CHD, however the plasma FB level was also influenced by sex, race, age, smoking, physical exercise, alcohol intake and hormone use. A genetic factor that governs the plasma FB level also seems to exist [4]. Both the FB level and the plasma viscosity (significantly influenced by FB concentration) are believed to be associated positively with total cholesterol, triglycerides, and the low density lipoproteins (LDL) and negatively associated with the high density lipoprotein (HDL) concentration. Smoking is the strongest known determinant of the plasma FB level in heathy persons [6]. Congenital hypofibrinogenemia and dysfibrinogenemia are asymptomatic, but only 20% of these patients were reported to have a tendency to develop thrombosis [7]. Acquired hypofibrinogenemias include therapeutic fibrinolysis, disseminated intravascular coagulation (DIC), liver failure (decreased synthetic function) and treatment with L-asparaginase, valproic acid or antithymocyte globulin [7].

The role of inflammation in the etiology and progression of CHD has been recognized and high white cell count is considered a risk factor [8, 9]. The activation of PMNL has been recognized as a major source of reactive oxygen species (ROS) release which is considerably increased during a chronic inflammation proces [2, 3]. The activation of polymorphonuclear leukocytes (PMNL) randomly influences several biological systems, that include blood coagulation and the nonspecific increase of acute phase proteins.

As FB and ceruloplasmin are acute phase proteins, their increased plasma level suggests an inflammatory response [1]. Another consequence of PMNL increased activity is plasminogen activation and release of plasmin which will trigger an additional fibrinogenolysis [10, 11]. As has been recognized [1,3], commonly used techniques for measuring fibrinogen degradation products (FDP) do not discriminate between FDP and fibrin degradation products, as both induce similar impairment of homeostatic functions [10]. Therefore, we studied the consequences of PMNL activation on the modulation of FB and FDP and subsequent chemiluminescent emission *in vitro* and *in vivo* in the plasma of patients suffering from CHD.

MATERIALS AND METHODS

Materials. Fibrinogen type I from human plasma, containing approximately 60% protein, fibrin, fibrinopeptide A, fibrinogen degradating compounds X and Y were obtained from Sigma (USA). They were dialyzed against 50mM Na-K phosphate buffer, pH 7.3. The concentrations measured at 280 nm by using a conversion factor of $E(1\%)_{280}$ =15. The purified protein solutions were adjusted to 5 mg/ml. AAPH (2.2' = azobis-2-aminodinopropane) was purchased from Wako (Chicago, IL). AAPH is a water soluble azo compound that thermally decomposes, leading to formation of aqueous peroxyl radical at a constant rate. The AAPH determination was performed under the conditions described by Gaziano et al [12]. This includes the procedure to isolate LDL by using removal of natural antioxidants by gel filtration and a column centrifugation.

Subject Selection. The blood used in this study was obtained from 62 men and 38 women cardiac catheterized at Fundeni Clinical Hospital. Coronary heart disease was defined as 80% stenosis of one of the major three coronary arteries as determined by angiography, according to the Budde et al procedure [13]. Of the total of 100 cardiac catheterized patients,

57.6% had 80-100% stenosis; 29.2%, 0% stenosis and the remainder had intermediate values between 30-80% of stenosis. The controls consisted of 68 age and sex matched persons apparently free of coronary heart disease.

Analytical Procedures. Fibrinogen and FDP were measured with a SIGMA Staphylococcal dumping test (proc. No. 850). For subjects with low FB levels, a second technique was added, based on calcium precipitation and subsequent protein measurement [14]. Platelet aggregation was determined by using a SIGMA kit (Proc. No. 885) based mainly on ADP reagent. For a quantitative expression, we used the proposed scale of respective kit. Ceruloplasmin was determined by its oxidase activity according to Schosinsky's method [15] and expresed in international units. The separation of cells from blood to obtain a suspension of PMNLs was accomplished by using a two step discontinuous Percol kit (SIGMA Chemical Co., St. Louis). Seventy percent of the PMNL suspension were neutrophils. The respective dilution of PMNLs to a concentration of $1x10^6$ cells/ml was performed by using Hanks' balancend salt solution (Gibco, Life Technol, Grand Island, N.Y.).

Activation of leukocytes. The CL technique recommended by Trush [16] was used for the measurement of PMNL activation by using zymosan^A (Sigma) opsonized with AB serum [17]. The activation of leukocytes was expressed as the stimulatory index (SI), the ratio of CL value at 12 minutes after zymosan addition as compared to an identical sample, without opsonized zymosan [17]. The CL emission produced by the activated PMNLs was measured by using a Beckman scintillation counter LS 3801 equipped with a single photon counting program.

Statistical Analysis. The results were reported as mean ± SD if not otherwise stated. Comparisons with the unmatched groups were made by Student's Impaired T-test. Statistical analysis was also undertaken with the use of Student's paired T-test after the groups were matched for age and sex. Individuals in the groups were arranged in a random order. Subjects with low fibrinogen level were matched to the specific control subjects. The data were transformed into logarithm to fit a Gaussian pattern. All calculations were performed by using a statistical package from BMDP Statistical Software Inc. No adjustment of the data was made for multiple comparisons, but the significance level was set at $P< 0.05$.

RESULTS

Our results indicate that PMNL modulated the biological activity of FB and FDP. *In vitro*, an interaction was found to exist between FB, FDP and activated PMNLs. Purified FB increased the concentration of zymosan-activated PMNLs. For a concentration correspon-ding to 200mg/dl (equivalent to a physiological level), FB did not influence the CL emission (Fig.1).

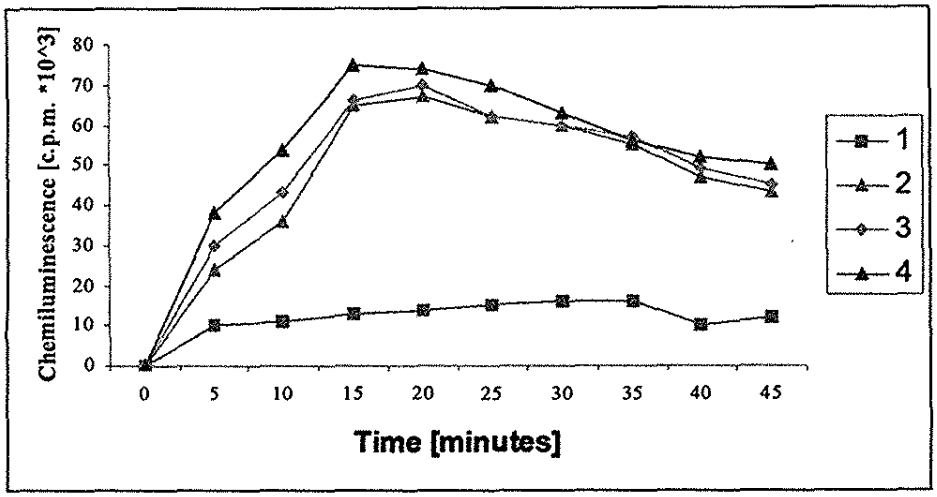

Fig. 1 The chemiluminescent response of the *in vitro* activation of polymorphonuclear leukocytes. The stimulation of LPMN was produced by opsonized zymosan (1 mg/ml). All variants have the same amount of viable PMNL ($2x10^6$ cell/ ml) obtained from the same normal donor.

Legend: (1) nonstimulated; (2) stimulated (s); (3) S + FB (2mg/ml); (4) S + FB (4mg/ml).

The reaction mixture contained PMNL suspension (100 µl $1x10^7$ cell/ml), 100 µl luminol 100 µM, the fibrinogen or related compounds in amounts already mentioned and Hanks nutritive medium (containing Mg and Ca) up to a final volume of 1.5 ml.

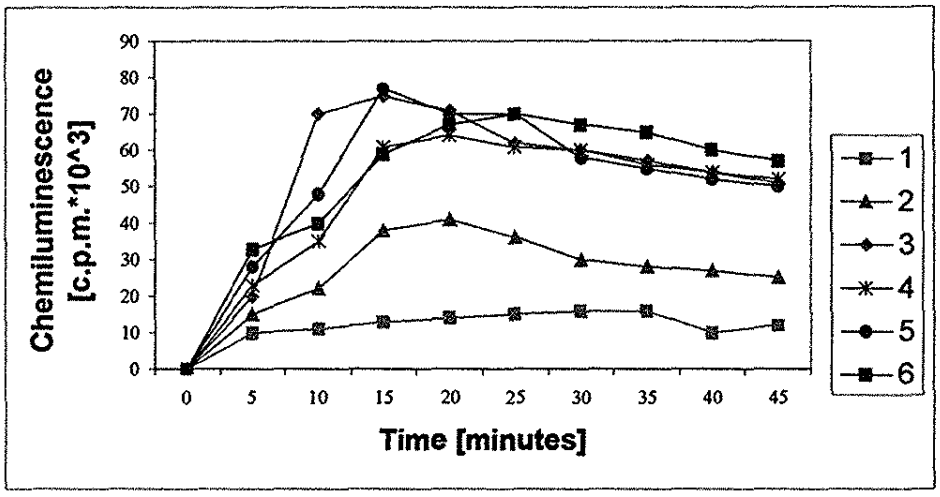

Fig. 2 The chemiluminescent response of the *in vitro* activation of polymorphonuclear leukocytes. The stimulation of PMNL was produced by opsonized zymosan (1mg/ml). All variants have the same amount of viable PMNL ($2x10^6$ cell/ml) obtained from the normal donor.

Legend: (1) nonstimulated; (2) stimulated (s); (3) fibrin (2mg/ml); (4) FDP-y(1mg/ml); (5) FDP-x (1mg/ml); (6) fibrinopeptide A (1mg/ml).

The reaction mixture contained PMNL suspension (100 µl $1x10^7$ cell/ml), 100 µl luminol 100 µM, the fibrinogen or its related compounds in amounts already mentioned and Hanks nutritive medium (containing Mg and Ca) up to a final volume of 1.5 ml.

At a corresponding 400 mg/dl level (equivalent to the upper physiological level), a significant increase in CL was observed. furthermore, under pathological conditions, the concentration of FDP may increase. As shown in Fig. 2, for a FDP concentration of 200 µg/ml, a significant increase of CL emission was found. The increasing order is stimulated PMNLs < FDP-y < FDP-x < fibrinopeptide A. Fibrin in a similar concentration partially inhibited the CL emission of activated PMNLs. FB and FDP may interfere with LDL oxidation caused by

activated PMNLs. Ample evidence supports such an action [10, 11].
As shown in Table 1, under the condition which favors LDL oxidation (Cu^{2+} and AAPH induced), purified FB acts significantly different, as a function of its concentration. Purified FB acted as a mild pro-oxidant for a 1 mg/ml concentration and as an AO for a 4mg/ml level.

Table 1. The effects of fibrinogen and related compounds on Cu^{2+} and AAPH-induced peroxidation of LDL.

Compounds	Lag Phase (min)	
	Cu^{2+}	AAPH-induced
Control	134± 17	72 ±8
+ Ascorbate 25 μmol/L	194± 16*	148 ±11*
+ Albumin 20 mg/ml (300 μM)	163± 14*	134 ±16*
+ Fibrinogen 1 mg/ml (6μ M)	102 ± 18*	61 ±7
+Fibrinogen 4 mg/ml (12μM)	175 ± 13*	137 ±15*
+ Fibrin 1 mg/ml	106 ± 12*	62 ±4
+ Fibrin 3 mg/ml	113 ±10	65 ±6
+ Fibrinopeptide A 1 mg/ml	104 ±8*	68 ±5
+ Fibrinopeptide A 3 mg/ml	112 ±13	74 ±7
+ Fibrinogen Degradation Product Y 1 mg/ml	116 ±15*	63 ±7
+ Fibrinogen Degradation Product X 1 mg/ml	132 ±11	75 ±9
+ Cumene Hydroperoxide 10 μM	76 ±8*	51 ±7*

LDL (500 μg/ml protein) was incubated with 3 μM $CuSO_4$ or with 3mM AAPH and 0.1 mM diethylene triamine penta acetic acid in the conditions already described (PBS medium). The absorbance at 234 was measured continuously and the lag phase, before absorbance increase, was calculated by using a molar extinction coefficient for conjugated dienes of $A_{234} = 2.95 \times 10^4$ cm^{-1}. * Significant differences as comparated with the control, for $P < 0.05$.

The FDPs act mainly as pro-oxidative, depending on their concentration. As for FB, its pro-oxidative action prevails at lower concentration. As shown in Table 2, 85% of the cardiac catheterized patiens had higher levels of FB, and FDP, ceruloplasmin and activation of PMNLs than age and sex matched non cardiac catheterized controls with no apparent CHD. For approximately 15% of the catheterized patiens, the FB level was lower than the physiological limit of 2g/L. However, these patients still had higher levels of FDP and higher levels of PMNL activation.

Table 2. Biochemical modification in subjects with low fibrinogen level as compared with catheterized patiens and matched controls.

	Non-Catheterized Patiens		Catheterized Patiens	
	Normal FB Matched Control	Low FB Matched Control	FB: High	FB: low
Fibrinogen g/L	2.78 ± 0.32	1.87 ± 0.34	3.85 ± 0.62*	1.52 ± 0.45
FDP (FB equiv), mg/L	217.91 ± 1.58	273.39 ± 18.3	389.47 ± 28.13**	576.48 ± 43.2**
Ceruloplasmin UI/L	108.31 ± 11.5	112.55 ± 17.3	218.46 ± 23.62*	195.06 ± 18.34*
Transaminase (GPT)(SFunits/ml)	21.42 ± 5.34	26.35 ± 5.15	25.38 ± 8.17	28.49 ± 7.35
Leuk. Phag. Stim. Index	24.62 ± 8.32	28.45 ± 9.32	58.36 ± 8.12**	64.73 ± 8.57**
Platelet aggregation (%)	88.7 ± 2.4	90.3 ± 3.4	95.6 ± 3.2*	23.4 ± 2.9

*Significant difference as compared with matched control for $P < 0.05$

** Significant difference as compared with matched control for $P < 0.01$.

DISCUSSION

This study emphasized the complex molecular modifications that occur in CHD. As acute phase proteins are constantly increased in inflamations, neoplasms and CHD, a relationship may exist with phagocytosing leukocytes and FB. Our results show that under normal physiological conditions the acute phase proteins, especially FB, did not influence significantly the activation of PMNLs. But under pathological conditions when these acute phase proteins are nonspecifically increased, especially FB may increase the biological activity of PMNLs as a function of its concentration.

Under physiological conditions, FB related compounds are found in small amounts which probably do not exert any significant action on activated PMNLs. FB was found increase in 85% of cardiac catheterized patiens, including the ones with 0% stenosis [14]. But 15% of these cardiac catheterized patiens exhibited low levels of FB. In these same 15% of patiens as FDP is increased, the FB decreased, although the activation of PMNL was still increased. A likely explanation may be the increased concomitant activation of thrombin and plasmin which may degradate increased amounts of FB [10, 11]. Such a mechanism is compatible with the existence of a chronic, inflammatory condition in these patients and unpredictable biological consequences. Our results indicate that the role of FB and FDP as an independent risk factor in CHD depends on the level of activated polymorphonuclear leukocytes in the plasma.

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