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Regular Article

Impaired fibrin gel permeability by high homocysteine levels

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ABSTRACT

Mechanisms involved in the relationship between hyperhomocysteinemia and thrombosis are still unclear. In previous reports we have shown that high homocysteine concentrations led to more compact and branched fibrin networks than controls. These clots showed an impaired lysis associated to their architecture. The aim of this study was to evaluate the effects of homocysteine on permeation of clots obtained from plasma and purified systems.

Fibrin gels were prepared with normal plasma incubated with homocysteine and, in the purified systems, with fibrinogen and factor XIII treated with the amino acid. Permeability constants (K_s) were determined through flow measurements.

Linear regression curve between K_s values and homocysteine levels in the plasmatic assays showed a negative correlation coefficient, $r = -0.997$ ($p = 0.003$). K_s of fibrin gels obtained from purified systems with fibrinogen incubated with homocysteine was $(7.07 \pm 0.27) \times 10^{-9}$ cm^2, control was $(11.40 \pm 0.37) \times 10^{-9}$ cm^2 ($n = 3$; $p < 0.01$). K_s of fibrin gels obtained with factor XIII treated with homocysteine was $(1.47 \pm 0.17) \times 10^{-9}$ cm^2, and control was $(3.31 \pm 0.31) \times 10^{-9}$ cm^2 ($n = 3$; $p < 0.01$).

Plasma incubated with high homocysteine concentrations produced fibrin clots significantly less permeable than controls in a dose dependent manner, and the results showed that fibrinogen and factor XIII were involved in that detrimental effect. These findings might explain the impaired fibrinolysis related to increased homocyteine levels and contribute to understanding the association between the amino acid and thrombosis.

Introduction

Hyperhomocysteinemia, the pathological increase of plasma homocysteine (Hcy) levels, is associated with increased risk of vascular occlusive disease such as arterial and venous thrombosis [1,2]. In past years a number of studies were undertaken to understand Hcy metabolism and the mechanisms of its toxicity [3]. Homocysteine, an amino acid with a thiol group, is an intermediate product in methionine metabolism. Genetic and acquired factors can impair the metabolic pathways, increasing plasma Hcy levels beyond normal. It has been proposed that homocysteinemia >12 μmol/l is related to a higher thrombosis risk [4,5].

In order to clarify the relationship between hyperhomocysteinemia and atherothrombosis a large number of studies *in vitro*, *ex vivo* and in animal models have been carried out and diverse mechanisms have been proposed. Most researchers have investigated the events preceding thrombus generation, but only a few have evaluated homocysteine's impact on the fibrin clot [6–8]. Quantitative and/or qualitative changes in the components involved in the fibrinformation can influence the fibrin network structure and consequently induce clinical disorders [9,10]. It has been reported that altered fibrin architecture is associated with coronary atherothrombosis and hypofibrinolysis, promoting recurrent ischemic events [11]. In order to characterize the fibrin clot, different physical properties are assessed, such as turbidity, permeation, viscoelasticity, rigidity, fibers size, and so on. In particular, fibrin gel porosity, stated as a permeability coefficient, would be in principle a parameter associated to fibrinolysis rate. It has been described that patients with hyperglycemia in acute myocardial infarction, patients with end-stage renal disease, individuals with some dysfibrinogenemias, etc, are associated with impaired plasma clot permeability and fibrinolysis [12–14]. Regarding Hcy, Undas *et al* have reported that homocysteinemia correlates significantly with fibrin clot permeation and fibrinolysis rate in human subjects [15].

We previously reported that fibrin networks generated in the presence of a high Hcy concentration were more compact and branched than controls and proved to be more resistant to fibrinolysis when urokinase-type plasminogen activator was used [6,8]. Therefore, our following aim in this subject was to evaluate the effect of high Hcy levels on the permeability of fibrin gels obtained from plasma and purified systems.

Abbreviations: Hcy, homocysteine; K_s, permeability or Darcy constant; PBS, phosphate-buffered solution; Fbg, fibrinogen; FXIII, factor XIII.

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Materials and Methods

Reagents

DL-homocysteine (Aldrich, Milwaukee, WI, USA), bovine thrombin (Sigma, St. Louis, MO, USA), human fibrinogen (Sigma Chemical St. Louis, MO, USA), highly purified plasma-derived pasteurized factor XIII (FXIII) concentrate (Fibrogammin P, Aventis Behring, Germany). All the other reagents were of analytical grade. Solutions of Hcy were prepared in phosphate buffered solution (PBS: NaCl, 137 mmol/l; KCl, 3 mmol/l; Na_2HPO_4, 15 mmol/l and KH_2PO_4, 1.5 mmol/l, pH 7.2). Fresh Hcy solutions were used in each assay.

Experimental procedure

Permeability assays were performed in plasmatic and purified systems.

Plasma fibrin

Normal pooled citrated plasma (platelet-depleted) was stored at −70 °C until further treatment. Aliquots of plasma pool were incubated (1 hour, 37 °C) with Hcy solutions (final concentrations: 50, 250 and 500 μmol/l) or PBS as control. Each sample, containing fibrinogen 3.2 g/l, was clotted into acrylic cylinders with thrombin and calcium chloride (final concentrations: 0.05 U/ml and 33 mmol/l, respectively). Care was taken to avoid leaving air bubbles in the plasmatic mixture. The gels obtained were left 2 hours at room temperature in a closed system. Then, acrylic columns were connected to a reservoir of degassed saline solution (NaCl 0.9% m/v) and were submerged into a water bath at 37 °C. The percolating solution was allowed to pass through the gels at five different hydrostatic pressures and the eluates were collected after periodic times. Permeation coefficient (Darcy constant, K_s) was calculated from the equation $K_s = (Q.\ L.\ \eta)/(t.\ A.\ \Delta p)$ [16], where Q is the volume of percolating solution flowing through the gel in time t (volumes were determined gravimetrically), L is the length of the fibrin gel, η is the viscosity of the percolating solution, A is the area of the gel perpendicular to the flow and ΔP is the differential pressure established by placing the reservoir at a specific height above the gel column [17]. Soluble substances in the fibrin gel were washed away at low pressure for 30 minutes before the assays. Measurements were performed in triplicate, at least at 5 different pressures.

Fibrin from purified components

Fibrinogen (Fbg) and FXIII solutions of physiological concentrations were separately incubated (1 hour, 37 °C) with Hcy (500 μmol/l) and PBS as control. In order to remove residual Hcy and put the samples under the same procedure, every solution incubated with Hcy or PBS, was ultrafiltered in Centricon-100 concentrators, 100,000 MW cut-off (Amicon, Inc, Beverly, MA, USA.). Concentrates were reconstituted to original volume with PBS. After three ultrafiltration cycles, total protein quantity was determined by Lowry method and Fbg and FXIII concentrations were adjusted to 1 g/l and 0.5 U/ml respectively. Treated fibrinogen (Fbg_{Hcy}), treated factor XIII ($FXIII_{Hcy}$) and their respective controls were obtained through this procedure.

Fibrin gels were yielded by adding thrombin (0.1 U/ml) and $CaCl_2$ (33 mmol/l) to different protein combinations: (*a*) Fbg_{Hcy} (1 g/l) plus FXIII untreated (0.5 U/ml) and (*b*) Fbg untreated (3 g/l) plus $FXIII_{Hcy}$ (0.5 U/ml). Controls of each system were performed. After 2 hours at room temperature, the flow rate through the gel was measured as described above. In order to obtain fibrin gels suitable enough to perform the permeability assays, it must be noted that each system required different Fbg concentrations.

Statistical analysis

Statistical analysis of the data was carried out using Statistix software for Windows 2.1 (Analytical Software, Tallahassee, FL, USA). Results were expressed as mean ± standard deviation (SD). Student *t* test was used to identify differences between Hcy-treated samples *versus* controls. A p value <0.05 was considered statistically significant. Linear regression was performed to evaluate the relationship between K_s values and Hcy concentrations and Pearson correlation coefficient was determined.

Results

Initially, we have developed permeability assays on fibrin gels obtained from plasma incubated with different Hcy concentrations ([Hcy]). Fig. 1 shows K_s values *versus* Hcy levels and the corresponding linear regression curve. K_s from Hcy-treated samples were significantly lower ($p<0.01$) than control. The strong negative coefficient correlation ($r=-0.997$; $p=0.003$) indicates that Hcy affects fibrin gels permeability in an inverse dose dependent manner.

Furthermore, permeability assays were performed on fibrin gels obtained from purified systems and K_s were calculated: (*a*) Fbg_{Hcy} and FXIII untreated resulted to be $(7.07\pm0.27)\times10^{-9}$ cm^2, while control (Fbg_{PBS}) was $(11.40\pm0.37)\times10^{-9}$ cm^2 ($n=3$; $p<0.01$). (*b*) K_s from fibrin gels obtained with Fbg untreated and $FXIII_{Hcy}$ resulted to be $(1.47\pm0.17)\times10^{-9}$ cm^2, and control ($FXIII_{PBS}$) was $(3.31\pm0.31)\times10^{-9}$ cm^2 ($n=3$; $p<0.01$). These results show that fibrin from Hcy treated proteins (Fbg_{Hcy} and $FXIII_{Hcy}$) turn out to be less permeable than their controls.

Although intra-assay K_s values were compared to the respective controls, inter-assay K_s can not be compared since different conditions were employed in each of the systems.

Discussion

The independent contribution of hyperhomocysteinemia to vascular occlusive disease has been vastly documented. This relationship is associated not only with severe but also with moderate and mild hyperhomocysteinemia. This metabolic abnormality, present in 5-10% of general population, is detectable in genetic and acquired deficiencies of enzymes and/or cofactors involved in Hcy metabolism [18,19].

In order to contribute to clarifying the mechanisms involved in the harmful effect of Hcy, we have previously investigated the effect of the amino acid on plasmatic fibrin structure, on the kinetics of fibrin-formation and on fibrin network lysis [6,8]. We have studied Hcy-associated plasmatic fibrin networks by electronic microscopy. They proved to be more branched and made up of thicker and shorter fibers than control, resulting in a more compact structure [6]. Although the

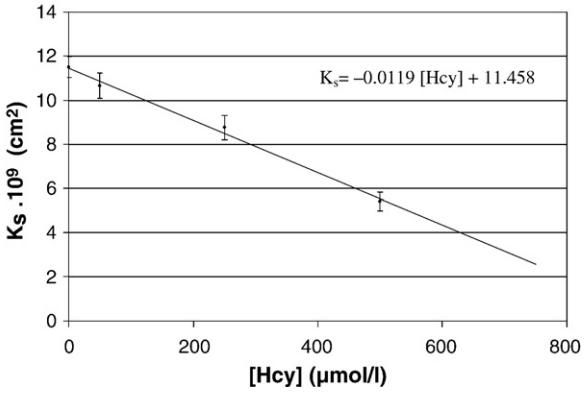

Fig. 1. Relationship between K_s values and Hcy levels ([Hcy]). K_s are expressed as mean ± SD ($n=3$). The linear regression curve is shown.