Homocysteine Binds to Human Plasma Fibronectin and Inhibits Its Interaction With Fibrin

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Objective—More than 70% of circulating homocysteine is disulfide-bonded to protein, but little is known about the specific proteins that bind homocysteine and their function as a consequence of homocysteine binding.

Methods and Results—When human plasma was incubated with [35S]L-homocysteine, most of the homocysteine bound to albumin. However, additional homocysteine-binding proteins were detected, and 1 of them comigrated with fibronectin. Treatment with 2-mercaptoethanol removed the bound homocysteine, demonstrating the involvement of disulfide bonding. In contrast, [35S]l-cysteine did not bind to fibronectin. Purified fibronectin bound ≈5 homocysteine molecules per fibronectin dimer. SDS-PAGE of a limited trypsin digestion of homocysteinylated fibronectin showed that several tryptic fragments contained [35S]homocysteine. Sequence analysis demonstrated that the fragments containing bound homocysteine had localized mainly to the C-terminal region, within and adjacent to the fibrin-binding domain. Homocysteinylation of fibronectin significantly inhibited its capacity to bind fibrin by 62% (P<0.005). In contrast, neither the binding of fibronectin to gelatin nor its capacity to serve as an attachment factor for aortic smooth muscle cells was affected.

Conclusions—These results suggest that homocysteine may alter normal thrombosis and delay or interfere with wound healing by impairing the interaction of fibronectin with fibrin. (Arterioscler Thromb Vasc Biol. 2002;22:●●●-●●●.)

Key Words: homocysteine | fibronectin | fibrin | atherosclerosis | thrombosis

Homocysteine is a sulfhydryl-containing amino acid formed during the metabolism of methionine. In healthy well-nourished individuals, homocysteine metabolism is efficient and regulated, and the concentration of plasma total homocysteine is usually ≤12 μmol/L.1 Inborn errors of homocysteine metabolism, drugs that interfere with homocysteine metabolism, deficiencies of folic acid, vitamin B12, and vitamin B6, and certain disease states, such as chronic renal failure, increase plasma total homocysteine levels.2 The homocystinurias are a heterogeneous group of autosomal recessive diseases caused by inborn errors of homocysteine metabolism.3–5 Patients with untreated homocystinuria have severe hyperhomocysteinemia (50 to 500 μmol/L) and life-threatening premature cardiovascular disease. Thrombosis, with or without embolism, is the major cause of death. Postmortem studies show multiple thrombotic occlusions and widespread premature atherosclerosis with intimal thickening. However, even mild hyperhomocysteinemia (15 to 25 μmol/L), regardless of the underlying cause, is a strong independent risk factor for occlusive vascular disease, as shown by numerous case-control and prospective studies involving thousands of subjects.1,6 The mechanisms of homocysteine-induced atherosclerosis and thrombosis have not been fully elucidated.

In normal individuals, >70% of circulating homocysteine is disulfide-bonded to plasma proteins.7,8 The primary carrier of disulfide-bound homocysteine is albumin,9–12 but other proteins with accessible cysteine residues are also potential carriers. The binding of homocysteine to cysteine residues may disrupt normal protein structure and impair or alter its function.13,14 We have previously shown that human plasma proteins have a high capacity to bind homocysteine in vitro.15 Albumin,10–12 factor V, and factor Va14 are the only plasma proteins that have been shown to bind homocysteine, and factor Va is the only plasma protein functionally altered by homocysteine.14 Small amounts of homocysteine thiolactone, a cyclic form of homocysteine, are also reported to enter the circulation.16 Because of its activated carbonyl group, homocysteine thiolactone can react with primary amines to form stable amide linkages with proteins, including numerous plasma proteins.10

Human fibronectin is a multifunctional glycoprotein encoded by the gene FN on chromosome 2q34. Fibronectin plays key roles in cell adhesion, cell migration, embryogen-
esis, differentiation, hemostasis, thrombosis, wound healing, and tissue remodeling. Distinct domains of fibronectin mediate its interactions with numerous proteins and macromolecules, including collagen, fibrin, heparin, and DNA. Fibronectin has free cysteine residues and numerous disulfide bonds that could interact with homocysteine via oxidative and/or thiol/disulfide exchange reactions. We hypothesized that homocysteine binds to fibronectin via disulfide linkage and that this binding may modulate fibronectin function.

In the present investigation, we show that homocysteine binds to several human plasma proteins, including fibronectin. This is the first study to demonstrate that homocysteine binds to fibronectin in vitro and that this binding results in a functional change, the inhibition of fibrin binding. This inhibition could lead to prolonged recovery from a thrombotic event and contribute to vascular occlusion.

Methods
Please see online data supplement (accessible at http://www.atvb.ahajournals.org) for materials and detailed methods concerning the preparation of \([^{35}S]l-homocysteine, \([^{35}S]l-cysteine, thiol binding to protein, microsequencing, assays for fibrin binding, gelatin binding and cell adhesion, and statistical analysis.

Results
Homocysteine and Cysteine Binding to Plasma Proteins and Purified Fibronectin
To identify the plasma proteins that bind homocysteine, normal human plasma was diluted 1:1 with TES buffer and incubated with 500 \(\mu\text{mol/L}\) homocysteine, followed by separation of the proteins by SDS-PAGE and visualization of the radioactive proteins with phosphorimaging (Figure 1A). Although most of the radioactivity in the plasma was associated with albumin, one of the other radioactive bands comigrated with purified human plasma fibronectin, which also bound to homocysteine (Figure 1A).

Figure 1. The binding of homocysteine and cysteine to plasma proteins and purified fibronectin. \([^{35}S]l-homocysteine and \([^{35}S]l-cysteine (500 \text{ \(\mu\text{mol/L}\)} final concentration) were incubated with 50\% human plasma in 0.05 \text{ mol/L TES buffer (pH 7.4)} and with human fibronectin (1 mg/mL in 0.05 \text{ mol/L TES buffer, pH 7.4}) for 5 hours at 37\^\circ\text{C}. After the incubation, protein was precipitated with 1.5 \text{ mol/L perchloric acid and solubilized with SDS-PAGE sample buffer. Aliquots were subjected to analysis by SDS-PAGE. The gels were stained with Coomassie blue, dried, and subjected to phosphorimaging. A, Binding of \([^{35}S]l-homocysteine. Lanes are as follows: 1 and 3, human plasma; 2 and 4, human plasma fibronectin. Lanes 1 and 2 are without reduction, and lanes 3 and 4 are with reduction by 2-mercaptoethanol. B, Binding of \([^{35}S]l-cysteine. Lanes are as follows: 1, human plasma; 2, human plasma fibronectin.}

The binding of homocysteine to fibronectin increased with increasing homocysteine concentration, as shown in Figure 2. In the presence of 500 \(\mu\text{mol/L}\) homocysteine, the binding reached equilibrium within 5 hours. A maximum of 5 mol
homocysteine was bound per mole of dimeric fibronectin (Figure 2).

**Localization of Bound Homocysteine Within Fibronectin**

To identify the region(s) of fibronectin to which homocysteine binds, an aliquot of fibronectin labeled with \[^{35}\text{S}\] L-homocysteine was subjected to limited trypsin digestion, and the tryptic fragments were separated by SDS-PAGE under nonreducing conditions. The radioactive bands were detected with phosphorimaging, as shown in Figure 3. Several, but not all, of the resulting protein fragments were radioactive. Labeled fragments with molecular masses of 50 and 30 kDa and a doublet at 34/35 kDa were excised from the gel, reduced, alkylated, and subjected to exhaustive trypsin digestion. The resulting peptides were sequenced by using LC/mass spectroscopy (MS). The peptides detected in these analyses are shown in online Table I (accessible at http://www.atvb.ahajournals.org). The majority of the identified peptides lie within and adjacent to the C-terminal fibrin-binding domain (Figure 4).

The 30-kDa band contained 10 peptides. Eight of these peptides were between residues 2150 and 2356, within and adjacent to the C-terminal fibrin-binding domain, and near a free cysteine sulfhydryl group (Figure 4). The remaining 2 peptides were from the N-terminus and contained amino acids 58 to 67 and 133 to 140, which are located in the N-terminal fibrin-binding domain. The 34/35-kDa doublet contained 17 fibronectin peptides. The 11 most abundant peptides were again located between amino acids 2150 and 2356, within and adjacent to the fibrin-binding domain. Five peptides containing 65 amino acids were from residues 1822 to 1910 (Figure 4). The last peptide was significantly less abundant and, again, contained residues 58 to 67 from the N-terminus. The 50-kDa fragment was present in the undigested and the digested fibronectin (Figure 3) and contained 7 fibronectin peptides containing 97 amino acids from near the C-terminus. These peptides represented residues 1286 to

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**Figure 2.** Stoichiometry of homocysteine (Hcy) binding to fibronectin. Ultrapure fibronectin (0.45 μmol/L) was incubated with either 100, 250, or 500 μmol/L \[^{35}\text{S}\] L-Hcy for various periods of time. Unbound Hcy was removed by acid precipitation of the fibronectin, followed by extensive washing. Bound Hcy was quantified by determining the radioactivity associated with the fibronectin pellet. Data are mean±SD (n=3).

**Figure 3.** Determination of fibronectin peptides containing bound \[^{35}\text{S}\] L-homocysteine. Human plasma fibronectin was incubated with \[^{35}\text{S}\] L-homocysteine, as described in Figure 1. The radiolabeled fibronectin was precipitated with perchloric acid and resuspended in TES buffer, and the pH was adjusted to neutral. The fibronectin was subjected to limited trypsin digestion for 6 hours at room temperature with 0.1% trypsin, and the digestion was stopped with 0.1 mmol/L phenylmethylsulfonyl fluoride. The resulting peptides were separated by SDS-PAGE on a 4% to 20% gradient gel under nonreducing conditions. The gel was stained with Coomassie blue, dried, and subjected to phosphorimaging. Lanes are as follows: 1, undigested fibronectin; 2, fibronectin subjected to limited trypsin digestion.
1301, 1788 to 1796, 1867 to 1910, and 2150 to 2176. Also present in this digest were peptides from BSA, a protein known to bind homocysteine, and a contaminant in the commercial fibronectin preparation used in the present experiment.

**Effect of Bound Homocysteine on Fibronectin Binding to Fibrin**

A direct binding assay was used to determine whether the binding of homocysteine in or near the C-terminal fibrin-binding domain affects the interaction of fibronectin with fibrin. Homocysteinylated fibronectin and control fibronectin were prepared by incubating fibronectin with or without 500 μmol/L L-homocysteine for 5 hours at 37°C. The amount of homocysteinylated fibronectin that bound to fibrin was only 38% of the amount of control fibronectin without bound homocysteine (P < 0.005), as shown in Figure 5.

**Interaction of Homocysteinylated Fibronectin With SMCs and Gelatin**

Because 1 of the free cysteine sulfhydryl groups of fibronectin lies near the cell-binding site (Figure 4), we examined the ability of homocysteinylated fibronectin to serve as a cell attachment protein for aortic smooth muscle cells (SMCs). The SMCs were preincubated with cycloheximide, which also was included in the wash and during the assay to prevent the production of endogenous fibronectin. Aortic SMCs attached as well to homocysteinylated fibronectin as they did to control fibronectin (108% relative to control attachment, n = 6). The SMCs did not attach to BSA. Although none of the radiolabeled fragments was localized near the gelatin-binding domain, the binding of fibronectin to gelatin is sensitive to reduction, so we examined the binding of homocysteinylated fibronectin to gelatin with a solid phase binding assay. Homocysteinylated fibronectin did not have a significant effect on gelatin binding (97% of control values, n = 5). These results indicate that the binding of homocysteine to fibronectin diminished its capacity to interact with fibrin but not with gelatin or cells and suggest that homocysteine is binding to select cysteine residues rather than randomly throughout the molecule. Sequencing of fibronectin fragments containing bound homocysteine confirms that the binding of homocysteine occurs primarily within and adjacent to the fibrin-binding domains.

**Discussion**

Fibronectin circulates as a dimer composed of 2 similar, but nonidentical, subunits joined by 2 disulfide bonds. Fibronectin has >60 cysteine residues per subunit, and most of these are involved in intramolecular disulfide bonding. Only 2 cysteine residues per chain exist as free sulfhydryls.

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**Figure 4.** Schematic diagram showing the various domains of fibronectin and the location of peptides obtained from sequencing of the tryptic fragments containing bound homocysteine. The domains are composed of types I, II, and III repeating units, as shown. Normal human plasma was incubated with [35S]-homocysteine and subjected to limited trypsin digestion. Three of the resulting peptides that were found to contain radioactive homocysteine were identified by exhaustive trypsin digestion, followed by sequencing with the use of LC/MS. The approximate locations of the sequenced peptides are shown. Peptides representing continuous stretches of sequence are indicated by only a single line.

**Figure 5.** Effect of bound homocysteine on the interaction of fibronectin (FN) with fibrin. Fibrinogen was dried onto the wells of a 96-well plate and subsequently converted to fibrin with thrombin, as described in online Methods (accessible at http://www.atvb.aha-journals.org). The wells were blocked with BSA, and then FN, with and without bound homocysteine, was added to the wells. The bound fibronectin was determined by ELISA. Data are mean ± SD and are expressed relative to control FN incubated with vehicle only (n = 5). FN-Hcy indicates homocysteinylated FN. *P < 0.005.
Homocysteine could conceivably interact with the free cysteine residues by direct oxidation or with the intramolecular and/or intermolecular cysteine disulfide bonds through thiol/disulfide exchange. The major findings of the present study are that homocysteine binds to plasma fibronectin by disulfide bonds and that homocysteineylation of fibronectin impairs its binding to fibrin. The results show that homocysteine binds to plasma fibronectin with a maximum of 5 mol homocysteine per mole of dimeric fibronectin. After incubation with radiolabeled homocysteine, fibronectin migrated on nonreducing SDS-PAGE gels with a molecular mass of the dimer, indicating that homocysteine did not disrupt the interchain disulfide bonds but, rather, bound to other cysteine residues.

Unlike homocysteine, cysteine did not bind to fibronectin. Although structurally similar, homocysteine and cysteine differ in their chemical reactivity.2 The sulfhydryl group of homocysteine is a stronger nucleophile than that of cysteine and is thus more reactive.25 Compared with cysteine, homocysteine has a higher affinity for cysteine-binding sites on plasma proteins in vitro and in vivo.23–25 Thus, homocysteine can replace protein-bound cysteine, but cysteine cannot replace protein-bound homocysteine.23–25 Furthermore, we recently demonstrated that when homocysteine and cysteine are simultaneously incubated with human albumin, ~4-fold more homocysteine than cysteine becomes protein bound.11

To identify the region(s) of fibronectin to which homocysteine binds, a limited trypsin digestion of fibronectin that had been labeled with radioactive homocysteine was carried out. Several of the resulting protein fragments that contained the radioactive homocysteine were sequenced by using LC/MS.

The LC/MS sequence analysis detected and characterized a relatively extensive series of peptides in each of the 3 radiolabeled bands. The majority of these peptides were mapped to the C-terminus of the protein sequence, within and adjacent to the C-terminal fibrin-binding domain. This region of the protein also contains a free cysteine residue that may be capable of binding homocysteine. Of the additional peptides that were detected in each of these analyses, several peptides in the 50-kDa band were mapped to albumin, a contaminant in the commercial fibronectin preparation that was used, and a protein known to bind homocysteine.10–12 The remaining peptides, detected in the 34/35-kDa band and the 30-kDa band, were 3 fibronectin peptides from an N-terminal region that is also a fibrin-binding domain. The presence of these additional peptides is most likely due to comigration in the nonreducing SDS-PAGE separation of the limited proteolysis products. Overall, however, the pattern of peptides that were detected in these radiolabeled bands clearly supports the C-terminal region of the fibronectin sequence as a major homocysteine-binding domain. Additional experiments were carried out to test the significance of homocysteine binding in this region.

Two fibrin-binding domains present in each subunit mediate the binding of fibronectin to fibrin.26 The first is located at the N-terminus and is the primary binding site. A second site is located near the C-terminus. This fibrin-binding domain is in a region of fibronectin containing numerous intrachain disulfide bonds.19,26 Williams and colleagues21,27 have shown that reduction of fibronectin by dithiothreitol reduces the interaction of fibronectin with fibrin, thereby demonstrating the importance of intact disulfide bonds for efficient interaction. This finding suggests that prolonged exposure of circulating fibronectin to select biological thiol agents may inhibit its interaction with fibrin in vivo. Our results demonstrate that homocysteineylation of fibronectin significantly impairs its interaction with fibrin. Homocysteine might be breaking crucial disulfide linkages via thiol/disulfide exchange or might be binding to the free cysteine residue near the fibrin-binding domain. However, the free sulphydryls of fibronectin are cryptic and are only readily accessible in the presence of chaotropic agents.28,29 The precise localization of bound homocysteine on fibronectin requires further study.

In addition to its interaction with fibrin, fibronectin binds to gelatin and serves as an attachment factor for numerous cell types.10 Homocysteine-ylated fibronectin was just as effective as control fibronectin as an adhesion protein for aortic SMCs. The presence of bound homocysteine also had little effect on the binding of fibronectin to gelatin. These results suggest that the binding of homocysteine to fibronectin has specific effects and that the major physiological consequence of homocysteine binding is a reduction in the capacity of fibronectin to bind fibrin.

The binding of fibronectin to fibrin is important in thrombosis and wound healing. Tissue injury produces a rapid induction of the clotting cascade and the formation of a provisional matrix, the major components of which are fibrin and fibronectin.30 These processes are carefully orchestrated, and dysregulation of the sequential steps involved in their progression impairs normal blood clotting and wound healing. As wound healing progresses, the fibrin/fibronectin-rich clot is replaced by a matrix rich in fibroblasts and blood vessels and, finally, by a collagen-rich scar. The provisional matrix serves as a substrate for the adhesion and migration of mesenchymal cells. Fibroblasts and endothelial cells migrate from the wound area to the provisional matrix as part of the transformation to granulation tissue.31 Fibronectin is primarily responsible for the adhesion, spreading, and migration of the invading cells.32–34 Corbett and Schwarzbauer35 have shown that fibronectin also significantly increases the retraction of the clot by nucleated cells.

It has been speculated that low levels of plasma fibronectin present in individuals suffering from starvation, shock, burns, trauma, infection, and certain metabolic diseases may impair wound healing.36 Malnourished rats with low plasma fibronectin have delayed wound healing. However, the intravenous administration of fibronectin to malnourished rats significantly improves wound healing, demonstrating the importance of plasma fibronectin.36 The impairment of fibrin binding by bound homocysteine may result in a deficiency of fibronectin in the provisional clot and subsequent delays in wound healing and clot resolution.

The formation of thrombi on atherosclerotic lesions can lead to occlusion, ischemia, and death. Numerous studies have shown a significant correlation between total plasma homocysteine levels and cardiovascular disease mortality.37–42 In a prospective study involving patients with cardiovascular disease, Nygard et al38 reported that 24.7% of the
patients with total homocysteine levels >15 μmol/L had died after 4 years compared with only 3.8% of the patients with total homocysteine levels <9 μmol/L. These reports indicate that homocysteine levels are strongly related to acute events resulting in death. Most acute ischemic cardiac syndromes, such as myocardial infarction, unstable angina, and sudden cardiac death, are the result of atherosclerotic plaque rupture and subsequent thrombosis.\(^\text{43–47}\) A delay in the resolution of the clot may lead to the presence of a prolonged thromboticogenic site, which may serve as a nidus for further platelet accumulation and clot formation.

Atherosclerosis and restenosis have long been viewed as a form of prolonged wound healing. Geary et al\(^\text{48}\) demonstrated that the spatial temporal pattern of events after angioplasty, including deposition of extracellular matrix, is quite analogous to healing wounds and culminates in luminal narrowing. In addition to the physical wounds caused by angioplasty, plaque fractures and plaque ruptures can also be viewed as “wounds,” which may eventually result in luminal narrowing as a result of normal prolonged wound healing.\(^\text{49}\) A failure of vascular SMCs to repair wounds adequately may play a part in plaque rupture and sudden death.\(^\text{49}\) Yee and Schwartz\(^\text{49}\) found that fibronectin present in the clot links fibrin and SMCs, and they demonstrated that plasma protease inhibitors are required to prevent degradation of fibronectin (Ikari et al\(^\text{49}\)). An inhibition of fibronectin binding to fibrin by homocysteine may lead to a deficiency of fibronectin in the clot. Thus, such a deficiency of fibronectin may contribute, in part, to inadequate wound repair, plaque rupture, and sudden death.

In a series of elegantly designed experiments, Sakai et al\(^\text{50}\) used conditional plasma fibronectin knockout mice to investigate the role of plasma fibronectin in transient focal ischemia in the brain. After ischemia and reperfusion, the control mice, but not the fibronectin-deficient mice, had abundant deposition of plasma fibronectin in the infarcts. Two and 7 days after ischemia, the mice lacking plasma fibronectin had infarction volumes ~35% larger than those of the control mice. These observations demonstrate that plasma fibronectin reduces the amount of affected tissue after ischemia and reperfusion. Fibronectin also accumulates in cardiac infarcts after episodes of ischemia and reperfusion and may play a key role in limiting the infarct size,\(^\text{52}\) but additional experiments are required to prove this. A deficiency of fibronectin in infarcts, because of its binding of homocysteine and reduced interaction with fibrin, could lead to larger infarcts and may, in part, explain why homocysteine is a risk factor for sudden cardiac death.

The present study is the first to demonstrate that homocysteine binds to circulating fibronectin and that homocysteine may impair the binding of fibronectin to fibrin. Additional studies to identify the specific cysteine residue(s) involved in homocysteine binding are in progress. Further studies on the mechanisms of impaired fibrin binding may provide additional information on the clinical relevance of homocysteinylated fibronectin.

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References

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