Homocysteine Enhances Neutrophil-Endothelial Interactions in Both Cultured Human Cells and Rats In Vivo

Nicholas P.B. Dudman, Suzanna E. Temple, Xue Wei Guo, Weiyu Fu, Michael A. Perry

Abstract—Despite intense investigation, mechanisms linking the development of occlusive vascular disease with elevated levels of homocysteine (HCY) are still unclear. The vascular endothelium plays a key role in regulating thrombogenesis and thrombolysis. We hypothesized that vascular lesions in individuals with elevated plasma HCY may be related to a dysfunction of the endothelium triggered by HCY. We investigated the effect of HCY on human neutrophil adhesion and migration through endothelial monolayers. We also examined the effect of HCY on leukocyte adhesion and migration in mesenteric venules of anesthetized rats. We found that pathophysiological concentrations of HCY in vitro induce increased adhesion between neutrophils and endothelial cells. This contact results in neutrophil migration across the endothelial layer, with concurrent damage and detachment of endothelial cells. In vivo, HCY infused in anesthetized rats caused parallel effects, increasing leukocyte adhesion to and extravasation from mesenteric venules. Our results suggest that extracellular H2O2, generated by adherent neutrophils and/or endothelial cells, is involved in the in vitro endothelial cell damage. The possibility exists that leukocyte-mediated changes in endothelial integrity and function may lead to the vascular disease seen in individuals with elevated plasma HCY. (Circ Res. 1999;84:409-416.)

Key Words: endothelium ■ occlusive vascular disease ■ homocystinuria ■ hyperhomocysteinemia ■ leukocyte

Homocysteine (HCY) has emerged as a recognized risk factor for occlusive vascular disease,1 after many case-control and prospective epidemiological studies. Family distribution surveys have revealed that hyperhomocysteinemia in vascular patients has a substantial familial component.2,3 At present, the reasons for the association between hyperhomocysteinemia and increased vascular risk are not entirely clear, despite numerous investigations that have illuminated some aspects.4

The concept that HCY may pathologically affect vascular endothelial function5 has been supported by the recognition of the key regulatory role of the endothelium in thrombosis and thrombolysis and of its role in controlling vascular smooth muscle activity.5 In vascular patients with hyperhomocysteinemia, the ubiquitous distribution of occlusions within the vascular tree and the fact that both thrombosis and accelerated arteriosclerosis occur suggest pathological changes in endothelial function. We considered that if the vascular endothelium behaved as if it were chronically inflamed, many of the thrombotic and occlusive vascular events could follow. Previously, we have shown that HCY does not act as an agonist, as does tumor necrosis factor-α (TNF-α),7 to induce inflammatory changes in endothelial function. We found that HCY had no effect on the human endothelial expression of leukocyte adhesion molecules or the endothelial production of prostacyclin.8,9 This led us, in our present study, to investigate whether HCY acted indirectly by activating leukocytes to interact with and induce pathological changes of function in the vascular endothelium. The investigation of these hypotheses has led to a detailed study of the influence of HCY on interactions between neutrophils and the vascular endothelium, both in vitro and in vivo.

Materials and Methods

Reagents

DMEM, HEPES buffer, penicillin, streptomycin, human serum (HS), and PBS were obtained from Flow. Human serum fibronectin, glutamine, and medium RPMI 1640 (modified) were purchased from Trace Biosciences Pty Ltd. L-HCY thiolactone (HCL), sodium heparin from bovine lung, catalase from human erythrocytes, superoxide dismutase, Triton X-100, phosphor myristate acetate (PMA), and human recombinant TNF-α were supplied by Sigma Chemical Co. Endothelial cell growth factor (EGCF) was purchased from Boehringer-Mannheim. FCS and trypsin-versene solution were obtained from the Commonwealth Serum Laboratories. Collagenase type 1 was purchased from ScimaR. Sodium [51Cr]chromate in sodium chloride solution (20 mCi/mL) was bought from Amersham Australia Pty Ltd. Lymphoprep and dextran T-500 were from Merck Co. IgG1 mouse monoclonal antibody (mAb) specific for intercellular adhesion molecule-1 (ICAM-1), E-selectin, and vascular adhesion molecule-1 (VCAM-1) were obtained from British Biotechnology and were documented as being functional for...
adhesion blockade. Fluorescein-conjugated sheep anti-mouse Ig was from Silenus (Hawthorn, Australia). mAb CD11b anti–Leu-15 PE, CD18 anti–LFA-1B FITC, CD62L anti–Leu-8 FITC with relevant negative antibodies, CAMFolio CD11b anti–MAC-1, and CD18 anti–LFA-1B were bought from Becton Dickinson Co.

Falcon tubes were obtained from Becton Dickinson Co. Costar Transwell tissue culture inserts were from Corning Costar Corp. Other tissue culture plastic was supplied by Nunc Co and by Flow Laboratories Co. Zetapore filters were bought from Gelman Sciences Pty Ltd. Human umbilical cords were obtained from newborns, full-term vaginal or cesarean births at Sutherland Hospital (Sydney, Australia).

L-HCY was prepared from l-homocysteine thiocelolate and, when tested by high-performance liquid chromatography, was free of homocysteine, other nitrinhydrin-positive contaminants, and UV-absorbing impurities. HCY in aqueous solution is readily lost by oxidation. The rate of loss of L-HCY (200 μmol/L) in DMEM plus 20% HS at 37°C was 87% within the first 40 minutes (results not shown). For this reason, we added HCY every hour in prolonged cell culture experiments. In parallel experiments involving exposure of cells to cysteine, we added this compound every hour as well. In cell culture studies, lipopolysaccharide (LPS) was eliminated from all media and solutions by Zetapore filtration.

**Preparation and Culture of Human Cells**

**Human Umbilical Vein Endothelial Cells (HUVECs)** Venous endothelial cells were isolated from human umbilical cords by standard methods. The cells, in tissue culture flasks precoated with human serum fibronectin, were cultured in DMEM containing ECGF from bovine brain (2 mg/mL), hepatitis (10 mg/mL), HEPES (15 mmol/L), penicillin (5×10^5 IU/L), streptomycin (50 μg/mL), NaHCO₃ (44 mmol/L), and 20% HS, in 5% CO₂/air. These cells were used in experiments up to passage 4. Confluence was monitored microscopically before use of cultured HUVECs in neutrophil attachment and migration assays and in HUVEC detachment and 51Cr release assays.

**Neutrophils**

Neutrophils were isolated from peripheral blood of healthy donors. The blood was collected in citrated tubes and mixed with 5% dextran at a ratio of 4:1, causing the erythrocytes to sediment. After 30 minutes at room temperature, the upper layer containing the buffy coat was layered over an equal volume of Lymphoprep and centrifuged for 25 minutes at 25°C with 650g. The erythrocytes in the resulting pellet were lysed with 10 mL cold 0.2% NaCl, returned to isotonicity with 1.6% NaCl, and centrifuged for 5 minutes, 25°C at 450g. Purified neutrophils were resuspended in RPMI 1640 plus 10% FCS. Neutrophils prepared in this way contained >95% neutrophils assessed by differential staining and were >95% viable Trypan Blue exclusion.

Radiolabeled neutrophils were prepared by incubating 0.5 mL of the resuspended cells in RPMI 1640 containing 2.5% HS with 10 μL of sodium [51Cr]chromate. After incubation at 37°C for 30 minutes with regular gentle agitation, the excess label was removed by washing 3 times, and the cells were resuspended in RPMI 1640 plus 2.5% or 10% FCS. In experiments involving coculture of neutrophils and HUVECs, the 2 types of cells were either separated by a Transwell membrane or cultured together in close contact. These 2 arrangements are defined respectively as “indirect coculture” and “direct coculture.” Endotoxic-free conditions were maintained throughout experimental procedures, tested by E-Toxate (Sigma).

**Immunofluorescence Flow Cytometry**

Neutrophils, after activation with HCY (200 μmol/L) in RPMI 1640 plus 10% FCS with gentle shaking for 30 minutes at 37°C, were washed twice with Dulbecco’s balanced salts solution containing 2.5% FCS and sodium azide. These cells were then incubated in this solution with fluorescent mAb toward CD11b, CD18, or L-selectin for 30 minutes at 4°C. Labeled cells, after further washing, were resuspended in the same modified salts solution supplemented with formaldehyde (0.5%). Antigen expression was analyzed on a fluorescence-activated cell sorter (FACS) using LYSIS software and expressed as the mean fluorescence intensity per cell corrected for background.

HUVECs, after indirect coculture with neutrophils, were washed, trypsinized (0.1% trypsin, 0.2% EDTA), and washed with undiluted FCS and then PBS containing 1% BSA and 0.02% sodium azide. These cells were labeled first with mAb specific for ICAM-1, E-selectin, or VCAM-1 and then after washing, with a fluorescein-conjugated sheep anti-mouse immunoglobulin. Antigen expression was analyzed as for neutrophils.

**Neutrophil Adhesion and Migration Assays**

HUVECs were cultured to confluence in their normal DMEM/20% HS on Costar Transwell inserts (6.5-mm diameter, 5-μm pore, type 3421). To initiate an experiment, the HUVEC-colonized Transwell inserts and the wells below the inserts were washed with RPMI 1640 plus 10% FCS. The inserts and wells were then incubated for 4 hours at 37°C with RPMI 1640/10% FCS containing either L-HCY (200 μmol/L) or TNF-α (100 U/mL), 0.1 mL added above the insert and 0.6 mL in the well. Every hour, fresh HCY was added to the upper compartment. The Transwell inserts were then washed with RPMI 1640 and transferred to new, clean wells. The lower compartment contained 0.6 mL RPMI 1640 plus 10 mmol/L HEPES plus 0.5% human serum albumin, and to the upper compartment above the HUVEC monolayer was added 0.1 mL of medium containing 1×10⁶ 51Cr-labeled neutrophils. In some experiments, neutrophils were incubated with antibodies to CD11b, CD18, or L-selectin, 5 μg/mL, at room temperature for 15 minutes, before coculture with HUVECs. The cells were then cocultured for 90 minutes at 37°C, after which the upper compartment was washed out twice with 0.1 mL RPMI 1640 to remove nonadherent neutrophils. The under surface of the insert was vigorously rinsed with ice-cold PBS/0.2% EDTA that was collected in the lower compartment. Cells still attached to the insert were lysed in 0.7 mL 0.5 mol/L NaOH, whereas cells that had either migrated into the lower compartment or had been dislodged by the saline wash were lysed by addition of Triton X-100 to 0.1%. These 2 fractions were counted for 51Cr to give measures of neutrophil adherence and migration, respectively, expressed as a percentage of total neutrophils added to the HUVEC monolayer.

TNF-α stimulates neutrophil adhesivity. This we confirmed, and TNF-α was used as a positive control in the present study, at levels shown to enhance adhesion. In another study, we used PMA (0.4 μmol/L) as a positive control to stimulate CD11b and CD18 expression on neutrophils.

**Damage and Detachment of HUVECs Mediated by HCY-Treated Neutrophils**

These assays used the method of Harlan et al. Primary or first-passage HUVECs were trypsinized and seeded on fibronectin-coated, 4-well, flat-bottom plates (2×10⁵ cells/well) and cultured to confluence (1×10⁶ cells/well). Sodium [51Cr]chromate was added to the wells (10 μCi/well) in the last 5 hours of this culture. HUVECs were then carefully washed with 5 successive exchanges of RPMI 1640 plus 10% FCS of 500 μL/well. Neutrophils were activated with L-HCY before coculture with HUVECs. The neutrophils were incubated with HCY (200 μmol/L) in RPMI 1640 plus 10% FCS with gentle shaking for 30 minutes at 37°C. Neutrophils were then washed twice with RPMI 1640 plus 10% FCS before experiments. These neutrophils were added to HUVECs, at 1×10⁶ cells in 200 μL/well, and incubated at 37°C in 5% CO₂/air for 3 hours. Endothelial cell leakage was then determined by measuring 51Cr released into 200 μL of cell-free conditioned medium, removed from each well with care so as not to disturb the monolayer. Then, 200 μL of fresh medium was added to the remaining 500 μL in each well. Detached endothelial cells were suspended by repeated gentle pipetting, and the total volume of conditioned medium containing suspended cells was removed to determine detachment. Residual attached cells were lysed in 500 μL 1 mol/L NaOH. All samples were counted on a 1261 gamma counter (LKB Wallac). 51Cr released...
Leukocyte-Endothelial Interactions in Anesthetized Rats

Rats were obtained from the Animal Breeding and Holding Unit, University of New South Wales, Sydney, Australia. All experiments involving rats were approved by the Animal Ethics Committee of the University of New South Wales and were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Rats were anesthetized and surgically prepared for intravital microscopy, and the observations during various treatments were essentially as described previously for the cat.\(^3\) Male Sprague-Dawley rats (110 to 160 g) were not fed for 24 hours before surgery and were sedated with halothane (ICI Pharmaceuticals) before intraperitoneal injection with the anesthetic inactin (120 mg/kg body weight). Tracheotomy was performed to facilitate breathing during experiments, and a rodent ventilator (model 7025, Ugo Basile) was used when necessary. The right carotid artery was cannulated and attached to a Spectramed TXX-R pressure transducer connected to a chart recorder (model 79d, Grass) to measure arterial blood pressure and heart rate. The jugular vein was cannulated for the injection of D,L-HCY.

Intravital Microscopy

After a midline abdominal incision was made, the rats were laid on their left side on a heating pad (37°C) on an adjustable Plexiglas microscope stage. A segment of intestine was exposed and its wall was draped with saline-soaked gauze to immobilize the gut and then covered with Saran Wrap. This exposed mesentery was superperfused with physiological buffer (pH 7.4) containing (in mmol/L) NaCl 131.1, KCl 4.7, MgSO\(_4\) 1.2, NaHCO\(_3\) 20, and CaCl\(_2\) 20 at 37°C and bubbled with 5% CO\(_2\)/95% N\(_2\).

The mesenteric microcirculation was transilluminated with a 12V, 100W DC-stabilized light source and observed using an Olympus microscope with a \(\times20\) objective lens. A video camera (JVC model TMD210P) and a videocassette recorder (Victor, BR-S600E) produced and recorded images. A video time-date generator projected time, date, and stopwatch functions onto the images. The final magnification was \(\times1600\).

Single, unbranched venules with initial diameters of 25 to 39 \(\mu\)m and a length of \(>150\) \(\mu\)m were selected for study. The number of rolling, adherent, and extravasated leukocytes was determined offline during playback of videotape images. Leukocytes moving slower than erythrocytes in the same vessel were defined as rolling, and rolling velocity was determined by the time required for a leukocyte to traverse 100 \(\mu\)m of a venule. A leukocyte was considered to be adherent if it remained stationary for \(>30\) seconds.\(^16\) The number of extravasated leukocytes recorded included all extravasated leukocytes in a field of view. The number of adherent or extravasated leukocytes observed at 60 or 90 minutes was corrected for the number observed at \(t_0\).

Experimental Protocol

In a preliminary experiment to establish a suitable dose of HCY administration, sham-operated rats were injected with HCY, and the plasma levels were measured at various times during and after treatment. After arterial pressure, red blood cell velocity, and vessel diameter had stabilized, the rats received D,L-HCY (5 mg/kg body weight) or PBS. Treatment was started by methods previously reported.\(^17\) Rats were then injected with 5 \(\mu\)g of a monoclonal anti-rat P-selectin (RMP-1) labeled with \(^{125}\)I and a nonbinding matched mAb (P23) labeled with \(^{131}\)I. Blood samples were taken at 2.5 and 5 minutes. The rats were then exsanguinated, vascularly perfused with buffer, and individual tissues were analyzed for the presence of labeled anti-P-selectin, after correction for any incomplete washout using the radioactivity of the \([^{131}\text{I}P23]\).\(^18\)

HCY as a Potential Chemoattractant for Neutrophils

The question of whether HCY was a chemoattractant for human neutrophils was tested using a micro-Bodten chamber.\(^19\) The chemotactic S100 protein CP10 and complement protein C5A were used as positive controls.

Statistical Methods

For cell culture experiments, results have been expressed as mean\(\pm\)SD. \(P\) values were calculated by unpaired Student \(t\) test. In experiments with anesthetized rats, results are expressed as mean\(\pm\)SEM. Comparison of results between HCY-injected rats and saline-injected rats was assessed using unpaired Student \(t\) test.

Results

Previously, we have shown that HCY increases human neutrophil adhesion not only to plastic wells but also to HUVECs.\(^8\) The possibility that independent activation of HUVECs by HCY could contribute to this enhanced neutrophil-HUVEC interaction was investigated. HUVEC monolayers on Transwell membranes were pretreated with HCY (200 \(\mu\)mol/L) and washed before being directly cocultured for 90 minutes with \(^{51}\text{Cr}\)-labeled neutrophils. HCY increased both the proportion of neutrophils that adhered to the membrane and the proportion of neutrophils that migrated through the HUVEC layer and the membrane (Figure 1), ending up in the well beneath the Transwell membrane. These changes in neutrophil behavior were inhibited by the presence of antibodies to the neutrophil docking protein complex CD11b/CD18 but not by antibodies to another neutrophil...
surface protein, L-selectin (Figure 2). The dose-response curves showed increased neutrophil adhesion and migration at the lowest HCY concentrations tested of 50 μmol/L (Figure 3). When the HUVECs were preincubated with L-cysteine rather than HCY at the same concentrations, there was little or no stimulation of neutrophil behavior, indicating selectivity for HCY in the activation of HUVECs. Total plasma cysteine concentrations for healthy adults were reported between 197 and 308 μmol/L (n=18; reference 20).

We tested whether the HCY-induced increase in neutrophil adhesion to and migration through HUVEC monolayers also occurred in vivo. Anesthetized rats were injected with HCY or saline, and the behavior of circulating leukocytes in mesenteric postcapillary venules was observed by intravital microscopy. In these studies, plasma HCY levels were monitored (Figure 4). We found that HCY decreased the rolling velocity of leukocytes (Figure 5) in a concentration-dependent manner and that the rolling velocity was lowest at 60 minutes. The number of leukocytes that stopped and adhered to the vascular wall increased significantly in the presence of HCY (Figure 6). Perhaps the most striking effect of HCY, however, was its influence on the number of leukocytes that migrated out of the venules and into the perivascular tissues. Whereas in saline-injected rats there was a small linear increase in the number of extravasated cells, the increase was substantially enhanced by HCY (Figure 7). These HCY-related effects in anesthetized rats were consistent with the idea that HCY was able to induce leukocyte-endothelial adhesion and transendothelial leukocyte migration both in vitro and in vivo. We repeatedly noted that injection of HCY in the rats caused no change in either mean arterial blood pressure or venular blood flow rate.

Although HCY has been shown not to induce increased endothelial expression of the surface adhesion molecules ICAM-1, E-selectin, and VCAM-1 in vitro, the possible effect of HCY on endothelial surface expression of P-selectin had not been tested. P-selectin can mediate early interactions between leukocytes and the endothelium in the developing
affinity between these cell types, which leads to inflammation. In the present study, HCY infusion did not lead to upregulation of P-selectin. For example, P-selectin expression in the heart tissue of control animals was 0.004 \pm 0.003% injected antibody bound per gram of tissue, whereas in HCY-treated animals, the binding was 0.002 \pm 0.003%. A similar lack of binding was observed in kidneys, skeletal muscle, gastrointestinal tract, and lungs.

We investigated whether these HCY-induced changes in neutrophil behavior could be harmful or pathogenic. When HCY-treated neutrophils were directly cocultured with \textsuperscript{51}CrO\textsubscript{4}\textsuperscript{-}-loaded HUVECs, we found that the effect of HCY was to increase both the leakage of \textsuperscript{51}Cr from the HUVECs into the conditioned medium and the detachment of HUVECs from their substrate (Figure 8). Catalase fully inhibited the HUVEC damage that normally followed exposure of these cells to HCY-treated neutrophils. However, catalase had no effect on spontaneous HUVEC detachment in the absence of neutrophils (data not shown). Superoxide dismutase did not inhibit HUVEC damage induced by HCY-treated neutrophils. Antibodies against CD11b and CD18 also inhibited endothelial detachment, although anti-L-selectin did not. Again, preincubation of neutrophils with L-cysteine rather than L-HCY at the same concentrations caused little or no activation of neutrophils in this assay system, indicating a specificity for HCY over cysteine in neutrophil activation. For example, the detachment of HUVECs in the presence of unexposed and 100 \textmu mol/L L-cysteine-exposed neutrophils was 5.37 \pm 0.86% and 5.38 \pm 1.14% (mean \pm SD), respectively. By contrast, HUVEC detachment in the presence of 100 \textmu mol/L L-HCY-exposed neutrophils was 9.48 \pm 1.55%, \( P < 0.01 \), with respect to both the unexposed neutrophils and the cysteine-exposed neutrophils.

Because we had found that HCY induced neutrophil adhesion in the absence of HUVECs, we studied whether

Figure 5. Effect of HCY injection (2 injections, each 5 mg/kg body weight at 0 and 60 minutes) on the leukocyte rolling velocity in rat mesenteric venules. The rolling velocity was determined for both a control group of rats, \( n=5 \) (open bars), and rats injected with HCY, \( n=7 \) (hatched bars). Values are mean \pm SEM. \( **P<0.01 \) compared with the control group at the same time.

Figure 6. Effect of HCY injection (2 injections, each 5 mg/kg body weight at 0 and 60 minutes) on leukocyte adhesion to the mesenteric wall in rats. Leukocyte adhesion was determined for both a control group of rats, \( n=5 \) (open bars), and rats injected twice with HCY, \( n=7 \) (hatched bars). Values are mean \pm SEM. \( *P<0.05 \) compared with the control group at the same time.

Figure 7. Effect of HCY injection (2 injections, each 5 mg/kg body weight at 0 and 60 minutes) on leukocyte emigration from the mesenteric venules. Leukocyte emigration was determined for both a control group of rats, \( n=5 \) ( ), and rats injected twice with HCY, \( n=7 \) ( ). Values are mean \pm SEM. \( *P<0.05 \) compared with the control group at the same time.

Figure 8. HCY-induced PMN-mediated HUVEC detachment. After 3 hours of coculture at 37°C of PMNs pretreated with 200 \textmu mol/L-L-HCY and \textsuperscript{51}Cr-loaded HUVECs, the detached HUVECs were resuspended and counted. Catalase, superoxide dismutase (SOD), or mAb against CD11b, CD18, and L-selectin were added to the PMNs before coculture commenced. Values are mean \pm SD; \( n=4 \). \( **P<0.01 \), \( ***P<0.001 \) compared with HUVEC/PMN; \( +++P<0.01 \), \( ++++P<0.001 \) compared with HUVEC/PMN/HCY.
purified neutrophils could be activated concurrently in other ways by HCY. Purified human peripheral blood neutrophils were preincubated with 200 μmol/L HCY for 30 minutes and then washed. When the rate at which [1-14C]glucose was converted to 14CO2 was assessed, we found that preincubation of neutrophils with HCY did not change this oxidation rate. Likewise, preincubation of neutrophils with HCY did not alter the rate of neutrophil release of superoxide, measured by a ferricyanide reduction assay. Measurement of the neutrophil expression of CD11b and CD18 antigens by FACS showed that preincubation of neutrophils with 200 μmol/L HCY had no significant influence on the quantitative expression of these antigens. By comparison, incubation with PMA (0.4 μmol/L) caused a >2-fold increase in each antigen.

We studied whether exposure to HCY induced neutrophils to release inflammatory cytokines capable of inducing an inflammatory response in HUVECs. Neutrophils were placed within Transwell containers and indirectly cocultured with HUVECs in tissue culture plastic wells whose bottom inner surfaces were covered with the HUVEC monolayer in the presence or absence of 200 μmol/L L-HCY. We expected that if HCY induced neutrophil release of an inflammatory agonist such as TNF-α, the agonist but not the neutrophils would diffuse through the Transwell membrane into the lower compartment, where contact with HUVECs would lead to upregulation of ICAM-1, VCAM-1, and E-selectin. FACS analysis showed that the presence of HCY in the coculture medium induced no such HUVEC surface changes.

In chemoattractant assays, both the chemotactic S100 protein CP10 and C5A provoked strong responses with purified human neutrophils, whereas HCY had no effect.

Discussion

We previously showed that exposure of purified human peripheral blood neutrophils to HCY caused them to bind to plastic alone or to HUVECs, with almost identical dose-response curves. Enhanced adhesion was evident at ≤10 μmol/L HCY, increasing progressively to plateau at ≥200 μmol/L (P<0.01). At this HCY concentration, neutrophil adhesion was increased ∼50%. For comparison, upper levels for normal human plasma total HCY and for mild, moderate, and severe hyperhomocysteinemia have been defined as 15, 30, and 100 μmol/L, respectively, whereas levels found in patients with untreated homocystinuria can be at least several hundred μmol/L.

These results showed clearly that HCY affected the behavior of neutrophils in the absence of other cell types. Our experiments reported in the present study in which HUVECs alone were exposed to HCY and then washed before coculture with untreated neutrophils strongly suggested that the HUVECs were themselves independently affected by HCY. The evidence shows that contact of the neutrophils with HCY-treated HUVECs induced more neutrophils to adhere. Increased migration of the neutrophils through the HUVEC-coated membrane followed. Very recently, HCY has been reported to enhance the endothelial release of the cytokines interleukin 8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1; reference 23). These findings raise the possibility that HCY could enhance endothelial-mediated adhesion of neutrophils (and monocytes) by releasing appropriate cytokines to stimulate the neutrophils and monocytes to increased binding. Our in vivo study showed that hyperhomocysteinemia had no effect on expression of P-selectin antigen in rats. By contrast, Morise et al used this same procedure in rats to show that oral indomethacin induced a 60% increase in P-selectin expression in gastric mucosa, evident at 1 and 3 hours after drug administration. Thus, our findings indicate that increased adhesion of neutrophils to endothelial cells, caused by exposure of the endothelial cells to HCY, is unlikely to result from upregulation of endothelial P-selectin.

We can rule out adventitious LPS as the cause of the changes noted in the present study in neutrophil and endothelial behavior associated with exposure to HCY for the following reasons. First, our media and other solutions were Zetapore-filtered to remove LPS. Second, increases in neutrophil expression of both CD11b and CD18 were induced by PMA under conditions in which HCY caused no change, showing that the neutrophils were capable of upregulating CD11b and CD18 if LPS had been present in the HCY preparation. Third, HUVECs were induced by TNF-α to increase expression of ICAM-1, VCAM-1, and E-selectin ≥7-fold under conditions in which HCY caused no increase, again indicating absence of LPS from the HCY preparation.

The increase in neutrophil adhesivity during exposure to HCY is also highly unlikely to be a response to HCY oxidation products in the medium. We showed that adding catalase during incubation of neutrophils with HCY did not alter the increase in adhesion of these cells either to plastic or to cultured endothelial cells. In addition, the medium chosen for neutrophil experiments was RPMI 1640, whose formulation contains no salts of the oxidation catalysts Fe and Cu. Also, the neutrophil experiments were carried out in medium containing 10% serum, which chelates traces of Fe and Cu and retards oxidation.

Our in vivo rat findings showed some strong parallels with the in vitro results. In the anesthetized rats, HCY caused significant decreases in the rolling velocity of leukocytes in postcapillary mesentery venules, whereas the number of leukocytes that were arrested along the luminal surface of the venules significantly increased. These observations imply that the adhesion between circulating leukocytes and the vascular endothelial cells increased during HCY infusion. The significant increase in the number of leukocytes that extravasated from the venules during HCY infusion also reflected the HCY-induced trans-HUVEC/membrane migration of neutrophils in vitro. The type of leukocyte that adhered and migrated in the rat mesentery venules was tentatively identified in the present study as neutrophils, on the basis of characteristic size and granular appearance. Histopathological studies with cats by one of our investigators (M.A.P., reference 25) have shown that after inflammatory stimulation, the leukocytes that extravasate from postcapillary venules are predominantly neutrophils, with a minor proportion of monocytes.
The question arises as to whether the HCY-induced adhesion of neutrophils to endothelial cells in vivo would be pathogenic. Our in vitro results show that when HCY-induced neutrophils adhered to HUVECs, the endothelial cells became sufficiently damaged to leak $^{51}$CrO4 and became detached from their extracellular substrate. This process is dependent on the presence of neutrophils pre-treated with HCY. Evidence from postmortem studies of patients with homocystinuria does not indicate widespread endothelial desquamation; yet, in vivo endothelium that is leaky or losing cells by detachment would most likely have other altered functions as well, some of which could lead to more general vascular pathology. Although HCY did not stimulate the redox machinery of isolated neutrophils, evidence from our antibody studies would be consistent with the idea that neutrophils docked with HUVECs in a CD11b/CD18-dependent process, after which either or both cell types released $\text{H}_2\text{O}_2$. The $\text{H}_2\text{O}_2$ appeared to damage HUVECs, because experimentally added catalase prevented the HUVEC $^{51}\text{Cr}$ leakage and detachment. Our finding that HCY treatment did not appear to induce neutrophils to release inflammatory cytokines, as assayed by adhesion molecule expression on HUVECs, would also be consistent with the idea that contact with HUVECs was required before neutrophils became offensive. The HUVEC damage and detachment induced by HCY-treated neutrophils could have resulted from the effects of $\text{H}_2\text{O}_2$ as previously documented, but it is also possible that neutrophil neutral proteases played a role, at least in the detachment process.

We have found that HCY enhances leukocyte-endothelial interactions at concentrations not far above normal physiological levels and well below the maximum concentration of free HCY found in the plasma of homocystinuria patients. Thus, neutrophil-endothelial adhesion is stimulated by HCY at levels as low as 10 $\mu\text{mol/L}$ and neutrophil migration is enhanced by HCY concentrations between 0 and 50 $\mu\text{mol/L}$ (Figure 3). In a recent report, Durand et al. found that hyperhomocysteinemia, induced by methionine loading in experimental rats, was associated with enhanced macrophage tissue factor expression and platelet aggregation. The increase in plasma HCY was of the order of only 5 to 20 $\mu\text{mol/L}$. The fact that HCY appears to have specific effects on the functioning of neutrophils, endothelial cells, macrophages, and platelets at concentrations not far above normal physiological levels suggests that HCY could play a normal regulatory role in the functioning of nonerythrocyte blood cells. In addition, by interacting with the endothelium, HCY could be involved in regulating the direction of leukocyte traffic past and through the endothelium.

In summary, HCY selectively induces changes in vitro in both neutrophils and HUVECs, leading to increased adhesion between the 2 cell types. The increased adhesion involves surface changes to the neutrophils, but no changes to the endothelial surface adhesion molecules ICAM-1, E-selectin, VCAM-1, and P-selectin have been found. Contact between the 2 cell types results in neutrophil migration across the endothelial layer and to damage and detachment of the HUVECs. In vivo, HCY infusion in anesthetized rats causes parallel effects, increasing leukocyte adhesion to and extravasation from mesentery venules. The evidence does not suggest that the HCY effects are mediated by neutrophil-released cytokines, but it does indicate that extracellular $\text{H}_2\text{O}_2$, perhaps generated by the neutrophils, is involved in the HUVEC damage. The possibility that HCY in humans could enhance the adhesion of leucocytes to the vascular endothelium and lead to leukocyte-mediated changes in endothelial integrity and function, ultimately resulting in thromboses and vascular lesions, has not been considered previously. We are currently exploring some mechanisms of this HCY-related biology.

Acknowledgments

This work was supported by a grant from the National Health and Medical Research Council of Australia. Special thanks to the staff of the labor wards of the Sutherland Hospital and the St George Hospital, Sydney, Australia, for their help in the collection of the umbilical cords. Many thanks to J. Gamble, Hanson Center for Cancer Research (SA), for assistance in the neutrophil adhesion assay and to K. Yang for help in the endothelial cell detachment study.

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Circ Res. 1999;84:409-416
doi: 10.1161/01.RES.84.4.409

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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