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Hyperhomocysteinemia increases intimal hyperplasia in a rat carotid endarterectomy model

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Purpose: **This preliminary study investigated the ability to elevate the serum homocysteine (H[e]) levels and investigated the increases in postoperative neointimal hyperplasia (IH) in an environment with hyperhomocysteinemia and the resultant restenosis in a rat carotid endarterectomy (CEA) model.**

Method: **The 9 rats for the control group were fed rat chow, and the 8 rats for the H(e) group were fed H(e)-supplemented rat chow for 2 weeks before and after CEA. The animals underwent anesthesia, and a left common CEA was performed. After 14 days, the serum H(e) levels were measured and the left carotid artery was harvested and elastin stained. Morphometric measurements were used to calculate the area of stenosis of the lumen. The mean and the standard deviation of the mean were determined. The 2 groups were compared with the Mann-Whitney test and a linear regression model. Three additional rats per group were studied, with carotid artery sectioning with double immunohistochemical staining for 5-bromodeoxyuridine (BrdU) and α–smooth muscle (α-SM) actin.**

Results: **The serum H(e) level in the H(e) group was 36.32 μmol/L ± 15.28, and in the control group the level was 5.53 μmol/L ± 2.06 ($P = .0007$). IH presented as percent lumen stenosis was 21.89% ± 4.82% in the H(e) group and 4.82% ± 1.64% in the control group ($P = .0007$). The linear regression model of the serum H(e) levels and the percent stenosis showed a linear relationship ($r^2 = .72$). The α-SM actin staining revealed that nearly all of the cells in the IH area were of smooth muscle or myofibroblast origin and that 10.1% ± 2.6% of the cells were stained for BrdU in the control group versus 23% ± 7.1% in the H(e) group. Also, 9.3% ± 2.6% of the cells in the IH area were stained for BrdU and for α-SM actin versus 19.1% ± 5.6% stained for both BrdU and α-SM actin in the H(e) group.**

Conclusion: **This is the first study to examine IH after CEA and hyperhomocysteinemia in rats. The study shows that the elevation of serum H(e) levels can be obtained by feeding rats modified diets with added H(e). The consistent elevation of serum H(e) levels was associated with more than 4 times the amount of IH after a CEA in a rat model. (J Vasc Surg 1998;28:909-18.)**

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Patients who are symptomatic with a >70% carotid stenosis who undergo carotid endarterectomy (CEA) have an improved prognosis as compared with those patients who undergo medical treatment, as documented by the North American Symptomatic Carotid Endarterectomy Trial.[1] Operative repair is not without potential complications, one of which is postoperative restenosis. The overall restenosis rate can be as high as 31%.[2] Recently, recurrent stenosis of >50% was found in nearly 11% of 409 arteries at a 42-month follow-up. Stenoses of >80%, including occlusion, developed in as much as 2.1% of these arteries. The cumulative 5-year ipsilateral stroke rate in patients with recurrent steno-

sis was 5.6%.[3] The causes of restenosis are neointimal hyperplasia (IH), recurrent atherosclerosis, arterial wall remodeling, and technical problems. In addition to CEA, IH is a complicating problem in other areas of artery manipulation, such as angioplasty, stenting, and venous arterial anastomosis for bypass graft procedures.[4] These lesions occur primarily as a result of smooth muscle cell proliferation and migration and result in restenosis.[5] Many theories exist regarding the pathophysiology of IH, but no overall scheme has emerged. This work studies the relationship between IH and homocysteine (H[e]). The results could lead to a clearer understanding of IH pathophysiology and potentially identify a treatable etiologic factor.

H(e), a non–protein–forming sulfur amino acid, is metabolized by remethylation to methionine or by transsulfuration to cysteine.[6] H(e) elevation may result from inherited disorders of enzyme activity or from enzyme cofactor deficiencies of cobalamin, folate, or pyridoxine, with subsequent interruption of H(e) metabolic pathways.[7] Clinically, hyperhomocysteinemia is an independent risk factor for accelerated occlusive vascular disease as concluded in a meta-analysis of 27 studies.[8] The Framingham Heart Study showed that the incidence rate of carotid disease was twice as high for patients with elevated plasma H(e) concentrations.[9] Elevated serum H(e) concentrations are found in patients with asymptomatic carotid artery disease[10] and in 32% of patients with progression of carotid artery disease to the next category of stenosis when followed up with noninvasive testing.[11] The association between plasma H(e) concentrations and >25% carotid artery stenosis is independent of known risk factors, and the risk of stenosis was elevated in subjects with H(e) concentrations of >11.4 μmol/L.[9] These studies show an association of elevated H(e) with the progression and sequelae of vascular disease. To our knowledge, no study investigates the relationship between the restenosis of CEA as a result of IH and the serum H(e) levels. We chose to investigate this relationship in a rat model.

This study was designed to investigate the ability to consistently elevate serum H(e) levels with dietary modification and then to use the diet in a rat endarterectomy model to determine the influence of hyperhomocysteinemia on IH. Our hypothesis was that hyperhomocysteinemia would increase the amount of postoperative IH and the amount of resultant lumen stenosis in a rat CEA model.

MATERIALS AND METHODS

Randomly numbered Sprague-Dawley rats (weight range, 325 to 350 g) were used for the study. A control group of 9 rats was given water ad libitum and fed a diet that was L-amino acid defined, choline and iron supplemented, and pelleted (Diet #518754, Dyets, Inc, Bethlehem, Pa). The H(e) group of 8 rats was given water ad libitum and was fed a diet that was L-amino acid defined, choline deficient, iron supplemented, and pelleted, without folic acid, methionine, or choline, and with 4.5 g/kg of DL-H(e) (Diet #518590, Dyets, Inc) for 2 weeks before operation. The control group and the H(e) group diets were matched for calories, protein content, vitamin mixtures, and minerals. However, the control group diet had choline (14.48 g/kg), methionine (1.7 g/kg), and folate (2 mg/kg), and the H(e) group diet was deficient in these compounds. Also, the H(e) group diet had an added 4.5 g/kg of DL-H(e). A full listing of dietary components can be found in Table I. The postoperative diets were the same as the preoperative diets.

Rat carotid endarterectomy. The animals underwent sedation with isoflurane and anesthesia with an intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (10 mg/kg). After adequate anesthesia was confirmed with the lack of response to hind paw stimulation, a subcutaneous injection of normal saline solution (10 mL/kg) was administered in the upper middle back, which served as a fluid bolus to compensate for any intraoperative blood loss. A midline cervical incision was made with a sterile technique and a dissecting microscope (SZ40, Olympus, Olympus America Inc, Melville, NY). The superficial muscles were divided, and the dissection was carried down to the level of the left carotid artery. The cervical nerves in the region of the artery were dissected free to preserve pharyngeal function to prevent postoperative respiratory compromise.

After adequate carotid artery exposure was acheived, proximal and distal control at the bifurcation (1.0 to 1.5 cm apart) was obtained with occlusion tourniquets fashioned from 4-0 silk suture and polyethylene 50 tubing. An arteriotomy was made with a corneal blade and was extended to 6 mm in length with micro-scissors. A 27-gauge needle was used to transversely score the endothelial cell layer and the media across the vessel in 2 parallel lines that were approximately 4 mm apart. The endothelial cell layer and the inner portion of the media then were removed with micro-forceps. The arteriotomy was closed with a running 10-0 monofilament nylon suture (MS/9, Ethilon, Ethicon, Inc, Somerville, NJ) with a tapered 3/8 circle needle (BV75-3, Ethicon, Inc). The distal tourniquet was removed

first to assess suture line hemostasis and was followed by the removal of the proximal occlusion tourniquet. A sterile cotton-tipped applicator was applied gently to any suture line bleeding until hemostasis was achieved. After endarterectomy, the carotid artery was assessed with a Doppler scan to confirm patency. The superficial muscle layer was closed with a running 4-0 absorbable suture, and the skin was closed with an interrupted suture. The rats were observed after surgery, and, when they recovered from the anesthesia, they were allowed to resume the same diet that they had before surgery. The operating investigator was blinded to the type of diet that each rat was receiving.

Tissue harvesting. Two weeks after surgery, the rats underwent anesthesia as before and the left carotid artery was exposed. The abdomen was opened at the midline, and the distal aorta and the inferior vena cava were exposed. Blood (3 mL) was withdrawn from the inferior vena cava for H(e) determination. The inferior vena cava was transected, and the aortic root was cannulated with an 18-gauge catheter. Normal saline solution was infused at 100 mm Hg until the vena cava effluent ran clear, and then a solution of 4% formaldehyde and 1% glutaraldehyde was infused at a constant pressure of 100 mm Hg in an equal volume to the saline infusion to complete the perfusion-fixation process. One-centimeter sections of the left carotid artery were dissected free and placed in 10% formalin.

Morphometric analysis. The arteries were paraffin blocked, sectioned, and elastin stained with Verhoeff's stain and van Geison's stain. Two sections per artery were taken from the middle one third of the segments after endarterectomy as identified by the proximal and distal limits of the continuous 10-0 nylon suture arteriotomy closure to standardize the region of sectioning. The elastin-stained slides were photographed, and the outline of the actual lumen (the inner limit of IH) and of the outer limit of IH were traced onto transparencies. The characteristic pattern of IH was used to identify the demarcation between the outer limits of IH and the media/adventitial layers. These outlines were digitized with a computer scanner (Hewlett Packard Scanjet 6100C, Palo Alto, Calif), and the morphometric area measurements were performed with the National Institutes of Health Software Image program, version 1.44 (Bethesda, Md). Because the arterial cross-sectional shape is noncircular as a result of processing distortion, the normalized area of the outer limit of IH was calculated on the basis of the circumference of this outer limit as described by Law et al.[12] The actual lumen area was measured. The difference between the 2 areas (outer limit of IH minus the actual lumen) was determined as the area of IH. Because arterial cross sections from different animals are not of constant diameter, the amount of IH was calculated as the ratio of the absolute area of IH to the outer limit of IH.[14] This ratio, presented as the percent lumen stenosis and as a main end point, represents the proportion of the lumen area occupied by IH and allows the comparison of the arterial cross sections of varying sizes.[12] The 2 sections from each vessel were traced, and the circumference and area measurements were performed by 2 blinded observers. Minimal variability was seen between observer measurements.

H(e) measurement. H(e) is oxidized in body fluids to the disulfides homocystine and cysteine-H(e). These disulfides are reduced, and protein-bound H(e) is released with tri-n-butylphosphine in dimethylformamide, which allows the measurement of the total plasma H(e) levels. After the thiols are labeled with a thiol-specific fluorogenic reagent, they are separated by a reverse-phase high-performance liquid chromatographic method with fluorescence detection. A 4-point standard curve is carried through the derivation procedure along with an internal standard, cysteamine, for quantitation.[13]

Immunohistochemistry. To aid in the determination of the cell line of the origin involved with the IH formation and to investigate whether the state of replication is increased in the H(e) group as compared with the control group, 3 additional rats per group were studied with double immunohistochemical staining for 5-bromodeoxyuridine (BrdU) and α–smooth muscle (α-SM) actin.[14] The rats were fed their respective diets for 2 weeks before CEA. At 24 and 48 hours before the rats were killed, 2 weeks after CEA, these animals were given 50 mg/kg intraperitoneal of BrdU (Sigma, St Louis, Mo). The carotid arteries were harvested and sectioned as previously described for the initial study groups.

The Vectastain Elite ABC Kit (Vector Laboratories, Inc, Burlingame, Calif) was used for the immunohistochemical staining. The sections underwent deparaffinization and were quenched with hydrogen peroxide in methanol, placed in 2 N HCl for 1 hour, then placed in 0.2% pepsin in 0.01 N HCl for 30 minutes, and finally blocked with 10% goat serum (Vector Laboratories, Inc). The sections were incubated first with BrdU primary antibody and with monoclonal mouse antibody (1:100, Sigma) for 2 hours, and then with biotinylated secondary goat antimouse antibody (1:400, Vector Laboratories, Inc) for 30 minutes before incubation in the ABC reagent. The sections

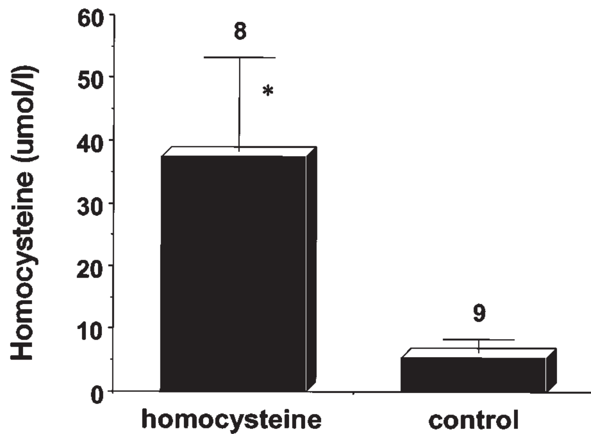

Fig 1. Serum homocysteine levels for homocysteine group (n = 8) and control group (n = 9; $*P = .0007$).

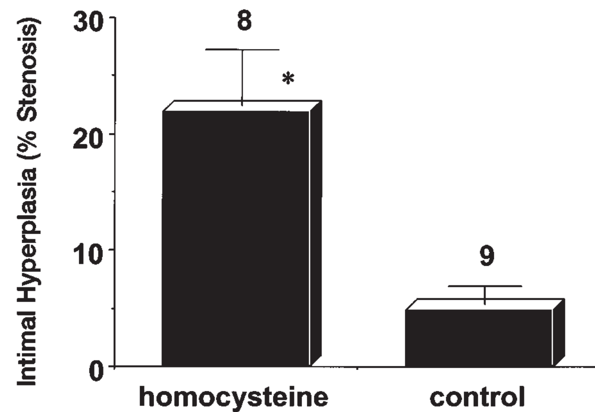

Fig 2. Amounts of intimal hyperplasia, expressed as percent stenosis, for homocysteine group (n = 8) and control group (n = 9; $*P = .0007$).

were developed with the peroxidase substrate solution, diaminobenzidine, for 6 minutes. The sections then were washed 3 times in phosphate buffered saline solution and blocked with 10% goat serum for 20 minutes. The sections were incubated for 2 hours with mouse α-SM actin primary antibody (1:400, Sigma) and then with goat anti-α–SM actin secondary antibody (1:400, Vector Laboratories, Inc) for 30 minutes. The staining for α-SM actin was completed with a VIP substrate kit (Vector Laboratories, Inc). Light green stain (1 minute) was used for counterstaining.

Statistical methods. All statistical analyses were performed with the GraphPAD InStat software (GraphPAD Software, Version 1.12a, Shreveport, La). The mean and the standard deviation of the mean (SD) for the H(e) levels and the percent stenosis was calculated for both the H(e) group and the control group. The H(e) levels and the percent stenosis for the H(e) group was compared with the control group with the Mann-Whitney test. The serum H(e) levels were compared with the percent stenosis with a linear regression model.

With the additional 3 animals for each group, the total number of cells that were stained for BrdU, for α-SM actin, and for both BrdU and α-SM actin in the area of IH were determined. The mean percentage of the cells stained were determined for each group: BrdU compared with the total number of cells, α-SM actin compared with the total number of cells, and BrdU and α-SM actin positive cells. The BrdU and α-SM actin data for the control versus the H(e) groups were compared with the Mann-Whitney test.

All of the animal care, the routine handling, the practices, and the procedures for this study, including the animal health monitoring, the diets, the cages, the environmental control, and the means of identification, met the standards described in the Animal Welfare Regulations and the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, publication 86-23, revised 1985). The protocol for the animal studies was approved by the Veterans Administration Animal Care and Use Committee (No. 7-96-2).

RESULTS

All of the animals survived the procedure except 1 rat in the H(e) group that died at the time of anesthetic administration before the operation. All of the carotid arteries that underwent endarterectomy were patent by gross inspection at the time of the harvest, and no arteries had evidence of thrombosis. The vessel measurements and the calculations for each individual study rat from the control and H(e) groups are listed in Table II. The mean and the SD for the circumference of the outer limit of IH was 1.86 mm ± 0.21 for the control group and 2.13 mm ± 0.14 for the H(e) group ($P = .008$). The mean and the SD for the area of the outer limit of IH in the control group was 0.28 mm^2 ± 0.06 and in the H(e) group was 0.36 mm^2 ± 0.05 ($P = .008$).

The mean and the SD for the H(e) levels in the H(e) group and the control group were calculated (Fig 1). The mean serum H(e) level in the H(e) group was 36.32 μmol/L ± 15.28. This was more than 6 times greater than the level for the control

Table I. Homocysteine and control group diet components

Ingredients	*Control group (g/kg)*	*Homocysteine group (g/kg)*
L-arginine	12.7	12.7
L-histidine	3.4	3.4
L-lysine-HCl	9.1	9.1
L-tyrosine	5.7	5.7
L-tryptophan	1.8	1.8
L-phenylalanine	7.3	7.3
L-methionine	**1.7**	**0.0**
L-cystine	3.7	3.7
L-threonine	4.6	4.6
L-leucine	10.5	10.5
L-isoleucine	6.1	6.1
L-valine	6.3	6.3
Glycine	6.2	6.2
L-proline	7.6	7.6
L-glutamic acid	28.9	28.9
L-alanine	5.1	5.1
L-aspartic acid	15.8	15.8
L-serine	7.2	7.2
Total L-amino acid	143.7	142.0
Cornstarch	100.0	100.0
Dextrin	100.0	100.0
Cellulose	50.0	50.0
Sucrose	392.19	403.87
Sodium bicarbonate	4.3	4.3
Corn oil	50.0	50.0
Primex	100.0	100.0
Salt mix (no Fe added)	35.0	35.0
Vitamin mix	**10.0 (with folate*)**	**10.0 (without folate)**
Choline bitartrate	**14.48**	**0.0**
Ferric citrate, USP	0.33	0.33
DL-homocysteine	**0.0**	**4.5**

**Folate*, 2.0 mg/10 mg vitamin mix; *USP,* United States Pharmacopeia.
Differences between diets are highlighted in bold print.

group, which was 5.53 µmol/L ± 2.06, with a *P* value of .0007.

The mean and the SD for the amount of IH, expressed as percent lumen stenosis, was determined for the H(e) and the control groups (Fig 2). The percent lumen stenosis was 21.89% ± 4.82% in the H(e) group and 4.82% ± 1.64% in the control group (*P* = .0007). This comparison showed more than a 4-fold increase in IH in the H(e) group when calculated as lumen stenosis. The data from the actual IH measurements (Table II) show an 8-fold increase in the IH area. However, the percent lumen stenosis is more accurate because it is a normalized value. This analysis of IH shows that approximately 78% of the lumen remains in the group with elevated H(e) levels versus 95% in the control group. Fig 3 is a representative photomicrograph of the elastin-stained sections that reveals minimal IH in the control group *(A1, A2)* as compared with a greater amount of IH formation in the H(e) group *(B1, B2)*. No evidence of thrombosis was seen in these specimens or in any of the specimens in both the control and the H(e) groups. Note that the internal elastic lamina is absent. A partial removal of the external elastic lamina is seen as a black staining line in *B2*, and the organized medial smooth muscle cells are indicative of the endarterectomy and the subsequent IH formation. The increased amount of IH is clearly evident as disorganized cellular proliferation and extracellular matrix deposition within the lumen of the H(e) group as compared with the decreased amount of reaction seen in the control group. As described below in the immunohistochemical data description, the cells in the IH contain α-SM actin and are primarily of smooth muscle or myofibroblast origin.[14,15] The nylon suture line is evident in the sections.

A linear regression model was used to compare the relationship between the serum H(e) levels and the percent stenosis. An analysis of the serum H(e) levels versus the percent stenosis showed a linear relationship with an r^2 value of 0.72 (Fig 4). As the serum H(e) levels increase, a corresponding increase is seen in the amount of IH and, thus, the lumen

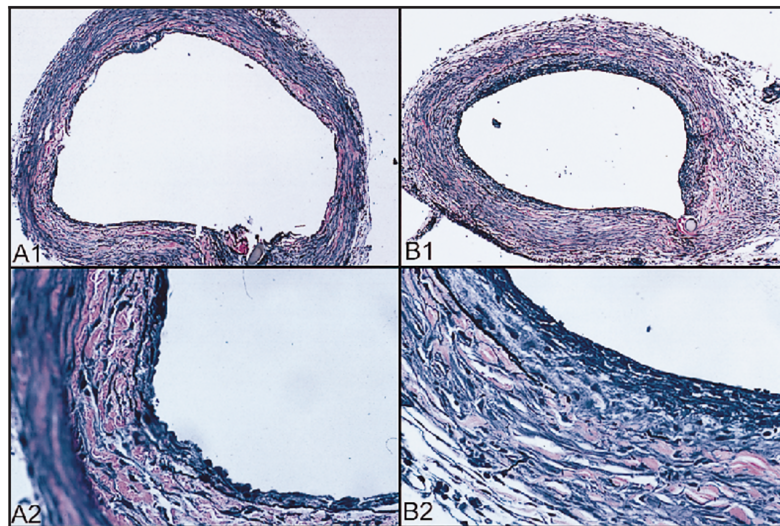

Fig 3. Photomicrograph of elastin-stained rat common carotid artery sections 2 weeks after CEA. Sections **A1** and **A2**, from an animal in the control group, show significantly less neointimal hyperplasia than sections **B1** and **B2**, from animals in the homocysteine group with elevated homocysteine levels. Magnification was ×10 (**A1** and **B1**) and ×40 (**A2** and **B2**). **A2** and **B2** are magnified sections of vessels seen in **A1** and **B1**. *Darker blue staining* near the lumen is identified as area of IH. Note lack of internal elastic lamina in **A2** and **B2** and partial removal of external elastic lamina in **B2**. Remaining external elastic lamina is seen as *thin black staining line*.

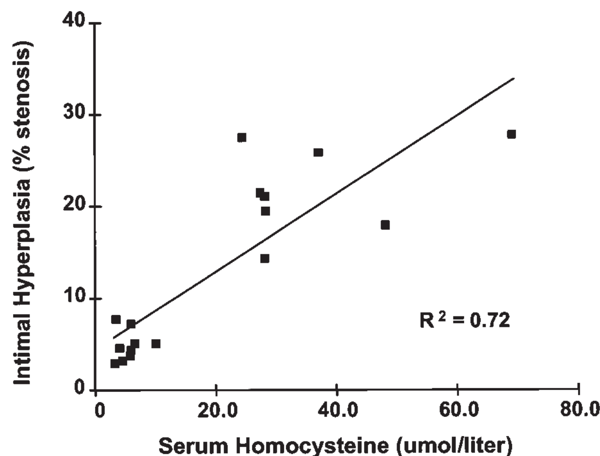

Fig 4. Linear regression model of serum homocysteine levels and percent stenosis ($y = 4.38 + 0.42x$; $df = 15$; $r^2 = 0.72$; $P = .0001$).

stenosis that is closely associated with the degree of H(e) elevation.

An examination of the double immunohistochemical staining in the additional 3 rats per group for BrdU and α-SM actin in the area of IH revealed the mean percentage of total cells stained for α-SM actin in the control group to be 99.0% ± 0.5% and in the H(e) group to be 95.0% ± 2.4%. The mean percentage of total cells in the IH area stained for BrdU was 10.1% ± 2.6% in the control group and 23.0% ± 7.1% in the H(e) group ($P = .04$). Finally, the mean percentage of cells stained for BrdU and α-SM actin was 9.3% ± 2.6% in the control group and 19.1% ± 5.6% in the H(e) group ($P = .05$).

DISCUSSION

Many studies have examined hyperhomocysteinemia and its association with various cardiovascular disease processes, such as peripheral, coronary, and carotid artery disease, and outcomes, such as stroke, myocardial infarction, and claudication.[16] However, to our knowledge, this is the first study to examine the increased IH development after CEA in an environment of hyperhomocysteinemia in rats. Studies that examined elevated serum H(e) have found an association with carotid artery thickening in patients who were asymptomatic[17] and symptomatic atherosclerotic disease involving the coronary, peripheral, and cerebral circulation,[16] but none have examined the development of IH after surgery. This preliminary study, although small, limited in scope, and focused on 1 H(e) dose and 1 post-CEA time point, is significant in that it may provide some insight into a causal factor for IH and it may provide

Table II. Vessel wall measurements for the homocysteine and control groups after CEA

Animal	*Area IH (mm²)*	*Circumference OL-IH (mm)*	*Area OL-IH (mm²)*	*IH/OL-IH × 100 (% stenosis)*	*H(e) level (µmol/L)*
H1	0.08	1.97	0.31	25.8	37.10
H2	0.09	2.30	0.42	21.4	27.30
H3	0.11	2.24	0.40	27.5	24.45
H4	0.07	2.21	0.39	17.9	48.06
H5	0.08	2.19	0.38	21.0	28.14
H6	0.07	2.13	0.36	19.4	28.30
H7	0.10	2.13	0.36	27.8	69.10
H8	0.04	1.88	0.28	14.3	28.09
Mean	0.08	2.13	0.36	21.9	36.32
SD	0.02	0.14	0.05	4.8	15.28
C1	0.02	1.81	0.26	7.7	3.47
C2	0.01	2.07	0.34	2.9	3.33
C3	0.01	1.97	0.31	3.2	4.58
C4	0.01	1.66	0.22	4.5	4.06
C5	0.01	1.70	0.23	4.3	6.02
C6	0.02	2.24	0.40	5.0	10.03
C7	0.01	1.59	0.20	5.0	6.55
C8	0.02	1.88	0.28	7.1	5.99
C9	0.01	1.84	0.27	3.7	5.75
Mean	0.01	1.86	0.28	4.8	5.53
SD	0.005	0.21	0.06	1.6	2.06
P value	.0007	.008	.008	.0007	.0007

IH, Neointimal hyperplasia; *OL-IH,* outer limit of neointimal hyperplasia; *H(e),* homocsyteine; *H1-H8,* homocysteine group; *C1-C9,* control group; *SD,* standard deviation of the mean.
P values compare mean values for H(e) group versus control group.

for a means of decreasing IH in various different scenarios that are encountered in vascular surgery.

Most rat carotid injury models involve a 2F balloon tip catheter introduced retrograde via the external carotid artery into the common carotid artery in an environment of normal serum H(e).[5,12,18,19] The catheter is passed through the carotid artery several times, which denudes the endothelial cell monolayer and stretches the vessel wall. Vessel injury occurs but is unlike that which occurs with human CEA operative conditions. A rat CEA with an arteriotomy, an intima and medial removal, and a suture closure has been shown to be feasible.[20] We modified this technique by obtaining vessel control with occlusion tourniquets and by using a continuous suture line. The following advantages are seen in this model: it more closely resembles the operation performed in humans; there is direct observable arterial wall manipulation and injury; and a continuous suture line is used, and there is no dilatation of the arterial wall. This model has proved to be reproducible—given the significant differences between the control and the H(e) groups—and more applicable and thus, may be applied to other areas of CEA investigation.

The interrelationship between vitamin status and plasma H(e) was first reported by Kang et al,[21] who demonstrated an inverse relationship between H(e) and plasma folate concentrations. The serum H(e) level has been shown modestly to increase 6.22 µmol/L after 2 weeks of administering a folate-deficient diet, without added H(e), to experimental animals versus control animals with a level of 4.85 µmol/L. Moreover, the homocysteinemia in rats with low normal levels of serum folate was similar to that observed in rats with subnormal folate levels, which suggests that the efficient remethylation of H(e) could not be attained in low normal levels of serum folate.[22] In light of these findings, this is the first study to use a diet designed with a deficiency of methionine and the methyl donors choline and folate and with the addition of H(e). In this way, the H(e) metabolic pathway is unable to proceed with the breakdown of H(e), which accumulates. This ensured a consistent significant supra physiologic elevation of serum H(e) similar to levels found in patients with homozygous metabolic enzyme defects. Once the elevated H(e) levels were achieved with this diet modification, its effects on IH were evaluated. The ability to produce such elevations with a simple dietary modification and without apparent confounding side effects provides for a useful tool to study increased H(e) lev-

els in different disease processes and treatment of the increased H(e) levels.

Another application for this model would be the continued study of the mechanisms by which H(e) contributes to vascular disease. The literature is replete with proposed mechanisms of action whereby H(e) promotes vascular injury and disease. McCully[23] demonstrated the direct toxic effect of H(e) on cultured endothelial cells, possibly as a result of the known cellular toxin H(e) thiolactone, by extracellular matrix modification of proteoglycans.[23]

Hyperhomocysteinemia may also affect normal wound healing and vascular repair. Vascular repair and associated neointimal formation have been associated traditionally with smooth muscle cell proliferation and migration from the media. In vivo studies have shown that oral administration of H(e) leads to significantly increased aortic cyclin–dependent kinase protein levels and to increased cellular proliferation as evaluated by BrdU incorporation. These increases paralleled H(e) levels and confirmed the role of hyperhomocysteinemia in the induction of vessel wall growth, proliferation, and thickening.[24,25] This effect of increased serum H(e) on vascular smooth muscle may play a role in explaining the mechanisms of action for the development of IH after CEA.

Recently, Shi et al[14] have shown that vascular injury repair and remodeling involves fibroblast activation, proliferation, migration, and differentiation to myofibroblasts as shown by the expression of α-SM actin. Remodeling has also been proposed as part of the explanation for lumenal decreases after balloon vessel injury.[14] An evaluation of the morphometric data (Table II) reveals that the circumference and the area of the outer limits of IH are significantly greater in the H(e) group versus the control group. A small circumference or area of the outer limit of IH would be the expected finding if remodeling was the explanation for the lumen stenosis. If this had been the case, the calculated percent stenosis of the vessels would have been even greater. Because the investigator was blinded, the measurement bias did not play a role in this finding. These results do not support the theory that contractile forces, associated with myofibroblasts, are attributing to the decrease in lumen area. However, remodeling may be present and may affect cellular migration and proliferation.

The double immunohistochemical staining data confirm that the primary cell type within the IH is α-SM actin positive and may either be smooth muscle or myofibroblasts. An examination of the BrdU data revealed that there were marginally significant increases in the replication of cells found in the H(e) group in an examination of the total population of cells and in those that were stained for α-SM actin. This suggests that there is an association between hyperhomocysteinemia and increased IH smooth muscle–myofibroblast migration and proliferation.

The close association between H(e) and coagulation and between the coagulation cascade and IH is well known and documented. One possible mechanism of action of increased IH in hyperhomocysteinemic rats is its role on coagulation factors. Although no detailed analysis of coagulation factors was undertaken in this model, we observed no thrombus formation grossly or at the time of sectioning in any carotid sample. Perhaps before the 2-week time point, some coagulation defect did occur that resulted in increased IH. However, this is only speculation at this time.

Normal serum H(e) levels have not been established for rats, and, for that matter, normal human serum H(e) levels are debatable. The upper limits of normal human serum H(e) levels were determined by Stampfer et al[26] to be as high as 15.8 μmol/L, and the risk for extracranial carotid artery stenosis was increased in subjects with H(e) concentrations as low as 11.4 μmol/L.[9] In our study, the control-group animals were administered diets with standard multivitamin preparations, including folate, B6, and B12. It is concluded that the mean H(e) level for the control group can be used as the "normal" serum H(e) level and can be used for comparison.

The consistent supra physiologic elevation of serum H(e) levels was associated with more than 4 times the amount of IH after a CEA as compared with those with normal H(e) levels (Fig 2). We anticipate that a longer postoperative period could possibly result in a more dramatic lumen diameter reduction, such that this lesion would be hemodynamically significant. Although other CEA models (balloon injury) appear to show a maximal lumen reduction effect at 2 weeks, we believe that, in the environment of continually elevated H(e), the IH will continue to encroach on the lumen. Also, there was a clear linear relationship between the serum H(e) level and the development of postoperative IH and restenosis at this time interval (Fig 4). We believe that this is a causal relationship because of the highly statistically significant results with a comparatively small sample size. In addition, the correlation analysis is highly indicative that an ever-increasing serum H(e) milieu results in a step-wise increase in lumen diameter reduction as a result of IH. The H(e) levels obtained in this study are nearly double those normally consid-

ered elevated and those in the range of patients with homozygous defects in cystathionine β-synthase. Studies with this rat CEA model and with a decreased amount of H(e) added to the diet are needed to attain elevated levels in line with those that are seen clinically. It is known that a deficiency of folate and other vitamins is associated with the accumulation of H(e) and that hyperhomocysteinemia is found not only in subjects with subnormal serum folate but also in those with low normal serum folate levels.[22] What is not known is at what level of folate does the lowest concentration of H(e) occur and how will this affect the formation of IH.

Clowes et al[5] in a balloon carotid artery injury model with no changes in H(e) levels have shown that total smooth muscle cells reach a maximum number at 2 weeks and then subsequently do not significantly change despite the continued proliferation of the luminal smooth muscle cells. Therefore, in this preliminary study, a period of 2 weeks was chosen to be the initial postoperative investigation of the effects of H(e) in a post-CEA model. Future investigations will include the lengthening of the postoperative period to more closely examine the long-term effect of hyperhomocysteinemia outside the perioperative period and to examine the nonoperative contralateral carotid artery controls from the study rats to investigate vessel wall changes in a nonoperative state. Dietary modulation is needed to establish the maximum benefit of dietary manipulation of H(e) levels and its effect on postoperative IH by repleting the folate in varying combinations.

The implication from this preliminary study is that, the lower the serum H(e) level, the less the progression of carotid artery IH after CEA. Our regression analysis data clearly showed this association between lower H(e) levels and minimal IH. The lowest limit of serum H(e) and associated decreased IH is yet to be established. This important factor, the decrease of H(e) from supra physiologic to physiologic levels and the reassessment of its role in IH, is the focus of further work. This study suggests that rats with increased H(e) levels are more prone to IH and subsequent restenosis after CEA.

An application to clinical scenarios of these preliminary animal model results of increased H(e) levels associated with increased IH after CEA would be premature. However, the results of this work could lead to a scenario where patients are screened before surgery for detection of hyperhomocysteinemia and possible correction with vitamin supplementation to decrease the incidence rate of recurrent stenosis. In humans, it is known that up to 40% of patients for strokes have elevated H(e) levels,[10,27] as compared with approximately 6% of the general population. What is not known is the percent of patients who undergo CEA, and who subsequently have restenosis, who have elevated or relatively high normal levels of serum H(e) and would potentially benefit from prophylactic treatment to normalize the H(e) level. Further clinical investigation will be required to establish the efficacy of such treatment.

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