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Promotion of vascular smooth muscle cell growth by homocysteine: A link to atherosclerosis

(DNA synthesis/cell cycle/cyclin/endothelial cells)

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ABSTRACT **Plasma homocysteine levels are elevated in 20–30% of all patients with premature atherosclerosis. Although elevated homocysteine levels have been recognized as an independent risk factor for myocardial infarction and stroke, the mechanism by which these elevated levels cause atherosclerosis is unknown. To understand the role of homocysteine in the pathogenesis of atherosclerosis, we examined the effect of homocysteine on the growth of both vascular smooth muscle cells and endothelial cells at concentrations similar to those observed in clinical studies. As little as 0.1 mM homocysteine caused a 25% increase in DNA synthesis, and homocysteine at 1 mM increased DNA synthesis by 4.5-fold in rat aortic smooth muscle cells (RASMC). In contrast, homocysteine caused a dose-dependent decrease in DNA synthesis in human umbilical vein endothelial cells. Homocysteine increased mRNA levels of cyclin D1 and cyclin A in RASMC by 3- and 15-fold, respectively, indicating that homocysteine induced the mRNA of cyclins important for the reentry of quiescent RASMC into the cell cycle. Furthermore, homocysteine promoted proliferation of quiescent RASMC, an effect markedly amplified by 2% serum. The growth-promoting effect of homocysteine on vascular smooth muscle cells, together with its inhibitory effect on endothelial cell growth, represents an important mechanism to explain homocysteine-induced atherosclerosis.**

Homocysteine, a sulfur-containing amino acid, is an intermediate metabolite of methionine. In patients with hereditary homocystinuria, deficiency in the enzyme cystathionine β-synthase leads to elevated levels of plasma homocysteine (hyperhomocysteinemia), which are believed to cause premature atherosclerosis and thrombosis (1–3). Although the incidence of homozygous homocystinuria is low (1 in 200,000), hyperhomocysteinemia (secondary to heterozygous homocystinuria, vitamin deficiencies, drugs, or other, as-yet-undetermined, causes) has been found in 20–30% of patients with premature atherosclerosis involving carotid, coronary, and peripheral arteries (4–8). Moreover, a recent large prospective study has defined hyperhomocysteinemia as an independent risk factor for myocardial infarction and stroke (9). Elevated plasma homocysteine levels in some patients can be reduced by vitamin supplements or dietary change (4, 10). Yet the mechanism whereby hyperhomocysteinemia induces atherosclerosis is unclear.

Proliferation of vascular smooth muscle cells is the most prominent hallmark of atherosclerosis (11, 12), and infusion of homocysteine into baboons causes myointimal cellular proliferation similar to the changes found in human atherosclerotic lesions (13). These data suggest that hyperhomocysteinemia induces proliferation of vascular smooth muscle cells in atherosclerotic lesions *in vivo*. However, it is not clear whether homocysteine causes smooth muscle cell proliferation directly or indirectly (through other cell types such as vascular endothelial cells, for example). Because the injured endothelium produces growth factors that act on neighboring smooth muscle cells to promote their proliferation, previous studies of homocysteine-induced atherosclerosis and thrombosis have focused on the effect of homocysteine on endothelial cells. High concentrations of homocysteine (5–10 mM) damage vascular endothelial cells *in vitro* (14). Homocysteine at 1–5 mM inhibits thrombomodulin secretion and protein C activation, reduces the number of cellular binding sites for tissue plasminogen activator, and impairs endothelium-derived vasorelaxation (15–18). It is difficult, however, to correlate these *in vitro* observations with clinical studies because atherosclerosis appears to be associated with homocysteine concentrations of 0.2–0.25 mM or lower in hyperhomocysteinemic patients (2, 3, 5). Moreover, the effect of homocysteine on vascular smooth muscle cell proliferation, in the absence of endothelial cells, has not been determined.

To better understand the role of elevated homocysteine levels in the pathogenesis of atherosclerosis, we examined the effect of homocysteine on the growth of vascular smooth muscle cells and endothelial cells at concentrations similar to those observed in clinical studies. We found that homocysteine increased DNA synthesis in rat aortic smooth muscle cells (RASMC) but decreased DNA synthesis in human umbilical vein endothelial cells (HUVEC). Homocysteine also induced cyclin D1 and cyclin A mRNA expression and promoted cell proliferation in RASMC.

MATERIALS AND METHODS

Cell Culture. RASMC were harvested from the thoracic aortae of male Sprague–Dawley rats (200–250 g) by enzymatic dissociation according to the method of Gunther *et al.* (19). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 mM Hepes (pH 7.4) in a 37°C, 5% CO_2, humidified incubator. Cells from passages 4–8 were used in the experiments. HUVEC (Clonetics, San Diego) were grown in M199 medium (GIBCO) containing 20% FCS, endothelial cell growth supplement (50 μg/ml), and heparin (100 μg/ml). Cells from passages 5–7 were used in the experiments.

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Abbreviations: RASMC, rat aortic smooth muscle cells; HUVEC, human umbilical vein endothelial cells; CS, calf serum; FCS, fetal calf serum; GAPDH, glyceraldehyde-phosphate dehydrogenase.

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[^{3}H]Thymidine Incorporation. RASMC in 24-well plates were made quiescent by incubation in DMEM with 0.4% calf serum (CS) for 72 hr (20) before addition of DL-homocysteine (Sigma). HUVEC in 12-well plates were incubated in M199 medium containing 20% FCS for 12 hr before addition of homocysteine. Cells were labeled with [*methyl*-^{3}H]thymidine (DuPont/NEN) at 1 μCi/ml (1 μCi = 37 kBq) during the last 3 hr of homocysteine treatment. After labeling, the cells were washed with Dulbecco's phosphate-buffered saline and fixed in cold 10% trichloroacetic acid, then washed with 95% ethanol. Incorporated [^{3}H]thymidine was extracted in 0.2 M NaOH and measured in a liquid scintillation counter. Values were expressed as the mean ± SEM from 6 wells from two separate experiments. Statistical analysis was performed by the Kruskal–Wallis test and significance was accepted at $P < 0.05$.

5-Bromodeoxyuridine (BrdUrd) Labeling. Subconfluent RASMC were plated on a chamber slide and then were made quiescent as described above. BrdUrd (Boehringer Mannheim) at 10 μM was added during the last 2 hr of homocysteine treatment and then allowed to react with alkaline phosphatase-conjugated antibodies to BrdUrd (21). After staining with chromogenic substrate, labeled nuclei (dark dots) were detected by light microscopy.

Amplification of Cyclin cDNA Fragments. Rat cyclin D1 and A cDNA fragments were amplified from mRNA of proliferating RASMC by the reverse transcription polymerase chain reaction (PCR) as described (22, 23). The sequences of primers for cyclin D1 (forward 5′-CCAGAATTCGARGTNTGYGARGARCA-3′ and reverse 5′-CCCGAATTCTCDATYTGYTCYTGRCA-3′) and cyclin A (forward 5′-CGTGGACTGGTTAGTTGA-3′ and reverse 5′-ATGGCAAATACTTGAGGT-3′) were based on the published mouse cyclin D1 and human cyclin A cDNA sequences (24, 25). The sequences of the PCR fragments were then determined by the dideoxynucleotide chain-termination method, with T7 DNA polymerase and an alkaline-denatured double-stranded plasmid DNA template (26).

RNA Extraction and RNA Blot Analysis. RASMC were made quiescent and then treated with 1 mM homocysteine for

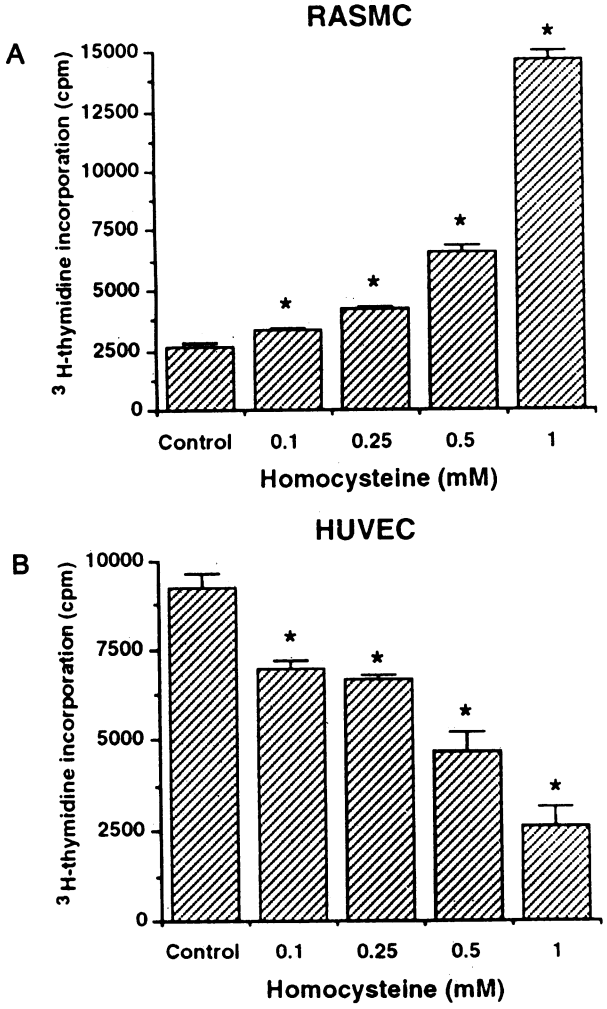

FIG. 1. Effect of homocysteine on DNA synthesis in RASMC and HUVEC. (*A*) Dose response of homocysteine-induced increases in [^{3}H]thymidine incorporation in RASMC. Confluent RASMC were made quiescent by incubation in DMEM with 0.4% CS for 72 hr. Then homocysteine was added at the indicated concentrations for 30 hr; [^{3}H]thymidine incorporation was measured during the last 3 hr. Values represent the mean ± SEM from six wells from two separate experiments. *, $P < 0.05$ vs. control. (*B*) Dose response of homocysteine-induced decreases in [^{3}H]thymidine incorporation in HUVEC. Confluent HUVEC were incubated in M199 medium containing 20% FCS without endothelial cell growth supplement for 12 hr before homocysteine was added. [^{3}H]Thymidine incorporation was measured and calculated as described for *A*.

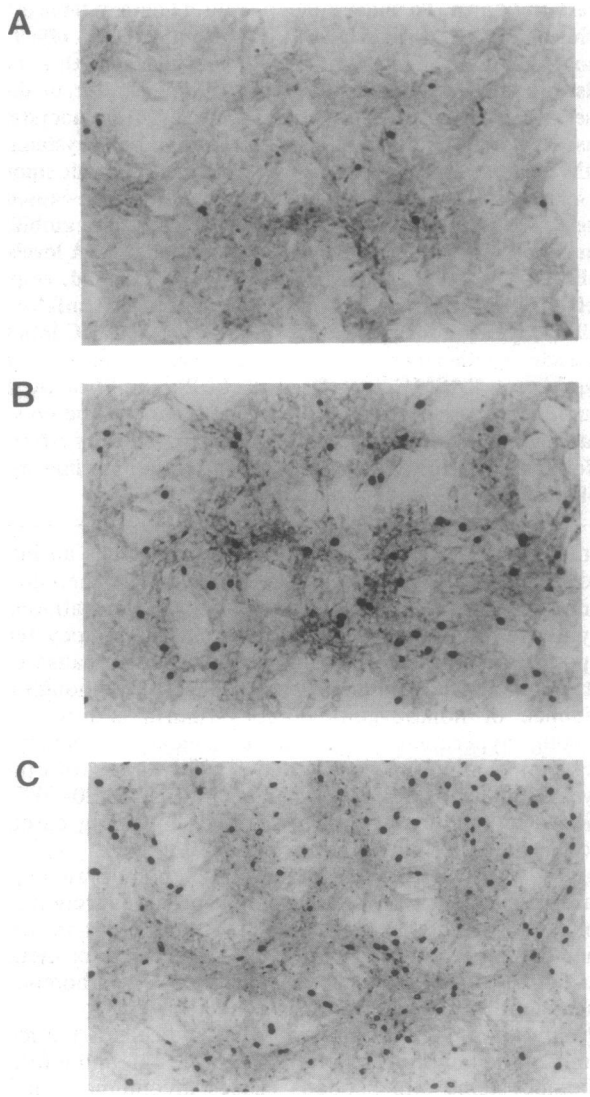

FIG. 2. Effect of homocysteine on BrdUrd labeling in RASMC. Subconfluent RASMC plated on a chamber slide were made quiescent and treated with control medium (0.4% CS), homocysteine (1 mM), or 10% CS for 30 hr. The dark dots (BrdUrd-labeled nuclei) represent cells undergoing active DNA synthesis. (*A*) RASMC treated with 0.4% CS. (*B*) RASMC treated with 1 mM homocysteine. (*C*) RASMC treated with 10% CS. (×40.)

various periods. Cellular RNA was extracted with guanidinium isothiocyanate and centrifuged through cesium chloride as described (22, 23). RNA blot analysis was performed with 10 μg of total RNA per lane. After electrophoresis the RNA was transferred to nitrocellulose filters, which were then hybridized to a ^{32}P-labeled rat cyclin probe and a glyceraldehyde-phosphate dehydrogenase (GAPDH) probe. The density of the signal from each band was measured in a PhosphorImager (Molecular Dynamics). To correct for differences in RNA loading, we divided the signal density for each RNA sample hybridized to the cyclin probe by that for each sample hybridized to the GAPDH probe.

Cell Proliferation Study. Quiescent RASMC were cultured in control medium (0.4% CS), 1 mM homocysteine, 2% CS, or 1 mM homocysteine plus 2% CS for various periods. Cells were counted in triplicate in a Coulter hemocytometer every 2 days. Medium and homocysteine were replaced every 2 days.

RESULTS AND DISCUSSION

Effect of Homocysteine on [^{3}H]Thymidine Incorporation in Vascular Cells. To better understand the role of elevated homocysteine levels in the pathogenesis of atherosclerosis, we examined the effect of homocysteine on DNA synthesis in both vascular smooth muscle cells and endothelial cells at concentrations similar to those observed in clinical studies. We made RASMC quiescent by incubating them in medium containing 0.4% CS for 72 hr (20). [^{3}H]Thymidine incorporation was then measured after the addition of homocysteine. Homocysteine increased [^{3}H]thymidine incorporation in RASMC in a dose-dependent manner (Fig. 1*A*). As little as 0.1 mM homocysteine

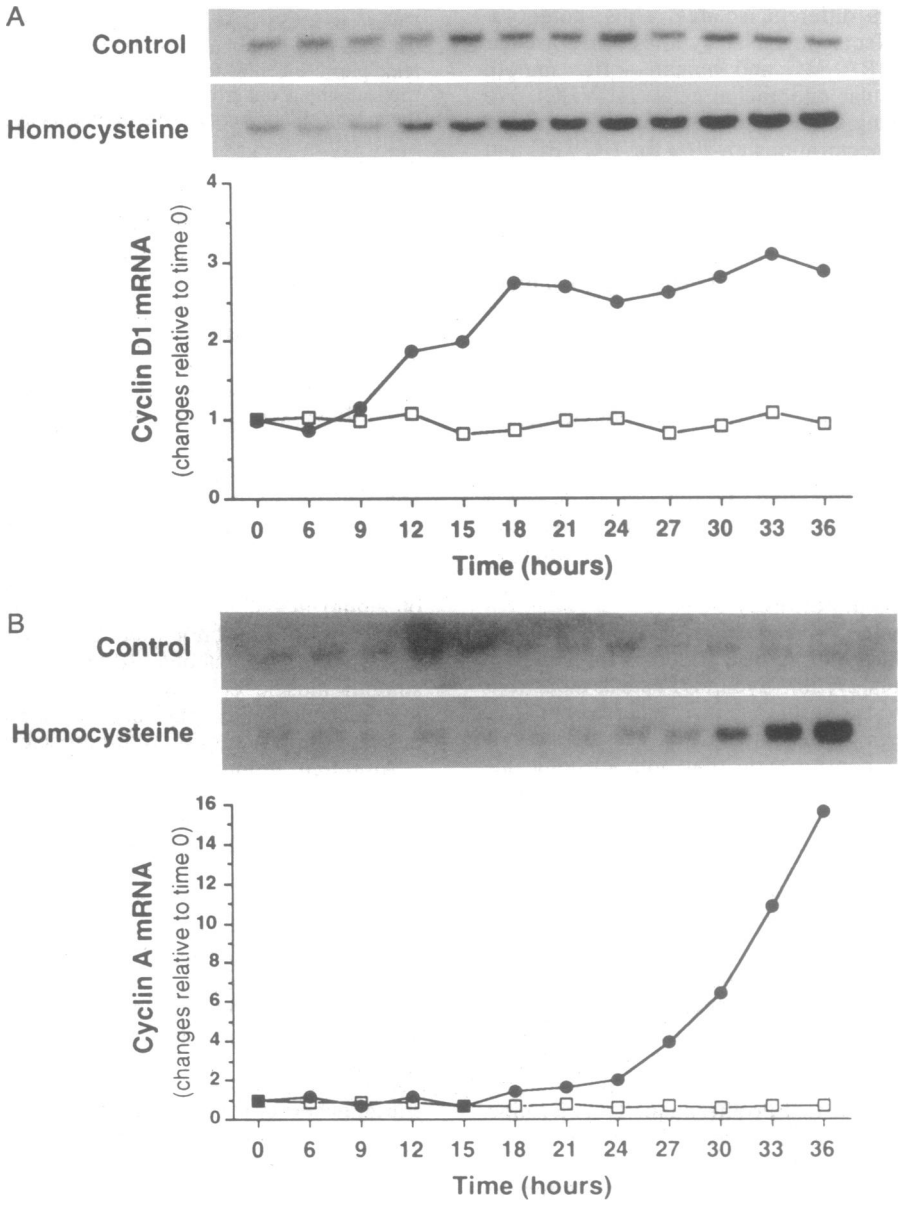

FIG. 3. Induction of cyclin D1 and cyclin A mRNAs by homocysteine in RASMC. Confluent RASMC were made quiescent and then treated with control medium (0.4% CS) or 1 mM homocysteine for the indicated times. Total cellular RNA was extracted at each point. RNA blot analysis was performed with 10 μg of total RNA per lane. After electrophoresis the RNA was transferred to nitrocellulose filters, which were then hybridized to a ^{32}P-labeled rat cyclin probe and a GAPDH probe. To correct for differences in RNA loading, the signal density for each RNA sample hybridized to the cyclin probe was divided by that for each sample hybridized to the GAPDH probe. The corrected value for each time point was then divided by that for time 0 and plotted against time. (*A*) RNA blot analysis of cyclin D1 mRNA. (*B*) RNA blot analysis of cyclin A mRNA. □, Control (0.4% CS); ●, 1 mM homocysteine.

caused a 25% increase in [^{3}H]thymidine incorporation, and homocysteine at 1 mM increased [^{3}H]thymidine incorporation by 4.5-fold. The increase in [^{3}H]thymidine incorporation began at 18 hr and peaked at about 30 hr, after which it declined (data not shown). Similar results were observed in RASMC prepared from different isolates. This effect was not species specific, as homocysteine also caused similar increases in [^{3}H]thymidine incorporation in human aortic smooth muscle cells (data not shown).

To test whether homocysteine affected DNA synthesis in vascular endothelial cells, we also treated HUVEC with homocysteine. In contrast with its stimulatory effect on [^{3}H]thymidine incorporation in RASMC, homocysteine inhibited [^{3}H]thymidine incorporation in HUVEC, again in a dose-dependent manner (Fig. 1*B*). As little as 0.1 mM homocysteine decreased [^{3}H]thymidine incorporation by 25%. At 1 mM, homocysteine reduced [^{3}H]thymidine incorporation by as much as 72% (Fig. 1*B*). Similar results were observed in HUVEC prepared from different isolates. Thus, homocysteine had opposing effects on DNA synthesis in vascular smooth muscle cells (RASMC and human aortic smooth muscle cells) and vascular endothelial cells (HUVEC). We confirmed these opposing effects in culture media containing serum in a range of concentrations (5–20% for HUVEC and 0.4–5% for RASMC, data not shown).

Effect of Homocysteine on BrdUrd Labeling. To determine the distribution of cells undergoing active DNA synthesis in response to homocysteine, we performed BrdUrd labeling in RASMC. In comparison with quiescent cells (Fig. 2*A*), there were approximately 4 times as many BrdUrd-labeled nuclei in RASMC treated with 1 mM homocysteine (Fig. 2*B*). This increase was consistent with the 4.5-fold increase in [^{3}H]thymidine incorporation induced by 1 mM homocysteine (Fig. 1*A*), and it confirmed the stimulatory effect of homocysteine on DNA synthesis in RASMC. In comparison with 10% CS, homocysteine induced approximately half as many BrdUrd-labeled nuclei (Fig. 2*C*), indicating that homocysteine was a potent stimulant of DNA synthesis in RASMC. Also, the BrdUrd-labeled nuclei were distributed evenly in homocysteine-treated RASMC, indicating that homocysteine increased DNA synthesis uniformly.

Effect of Homocysteine on Induction of Cyclin mRNA. Cyclins are important regulators of cell cycles triggered by growth factors or serum (27, 28). Cyclin D1 acts as a positive growth regulator during the early G_1 phase, whereas cyclin A has an important role in the S and G_2/M phases of the cell cycle. As a molecular confirmation that homocysteine induced quiescent RASMC to reenter the cell cycle, we measured cyclin D1 and A mRNA levels after treating RASMC with 1 mM homocysteine. Induction of cyclin D1 mRNA occurred 12 hr after the addition of homocysteine and plateaued at 3 times baseline between 18 and 36 hr (Fig. 3*A*). This induction of cyclin D1 mRNA at 12 hr preceded the increase in [^{3}H]thymidine incorporation at 18 hr. In contrast, induction of cyclin A mRNA did not occur until 27 hr after the addition of homocysteine, and it reached a level of 15 times baseline at 36 hr (Fig. 3*B*). These results indicate that homocysteine does induce the mRNA of cyclins important for the reentry of quiescent cells into the cell cycle.

Effect of Homocysteine on Cell Proliferation. To determine whether homocysteine-induced increases in DNA synthesis and cyclin mRNA levels were followed by cell division, we also counted the number of RASMC 2, 4, and 6 days after the addition of 1 mM homocysteine. Although homocysteine did not affect cell number significantly at day 2, there were significant and time-dependent increases in cell number at days 4 and 6 (Fig. 4). By day 6, homocysteine increased cell number by 38%. To determine whether serum altered homocysteine's effect on RASMC proliferation, we also treated RASMC with 2% CS or homocysteine plus 2% CS. Although

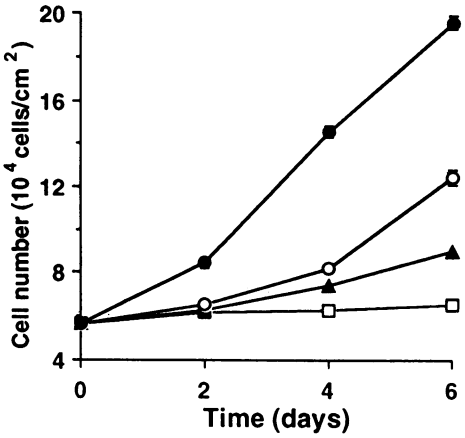

FIG. 4. Effect of homocysteine on the proliferation of RASMC. Quiescent RASMC were cultured in medium containing 0.4% CS (□), 0.4% CS plus 1 mM homocysteine (▲), 2% CS (○), or 1 mM homocysteine plus 2% CS (●) for the indicated periods. Cells were counted in triplicate in a Coulter hemocytometer every 2 days. Values represent the mean cell numbers from experiments in triplicate.

2% CS failed to increase cell number, homocysteine plus 2% CS increased cell number by 37% at day 2 (Fig. 4). This synergistic effect of 1 mM homocysteine and serum on RASMC proliferation persisted through days 4 and 6. Moreover, this effect was also observed at lower concentrations of homocysteine. Homocysteine at 0.25 mM did not change cell number significantly at day 4. However, in comparison with 2% CS alone, 0.25 mM homocysteine plus 2% CS caused a greater than 2-fold increase in cell number (data not shown).

We have shown that homocysteine increases DNA synthesis in vascular smooth muscle cells and induces them to proliferate. Especially interesting is its opposite effect on DNA synthesis in endothelial cells. At the same clinically relevant levels that homocysteine increased DNA synthesis in smooth muscle cells, it decreased DNA synthesis in endothelial cells. This does not appear to be a universal effect of amino acids, since leucine and methionine, at similar concentrations, did not affect the DNA synthesis of the two cell types (data not shown). By promoting the proliferation of smooth muscle cells while impeding the regeneration of injured endothelial cells, homocysteine may initiate or accelerate the progression of atherosclerosis.

Although homocysteine alone does induce quiescent RASMC to reenter the cell cycle and proliferate, homocysteine also interacts with serum in a synergistic manner to promote the proliferation of RASMC. Therefore it is likely that homocysteine interacts with other growth factors or cytokines present in atherosclerotic lesions (11, 18) to promote the growth of smooth muscle cells during atherogenesis. The growth-promoting effect of homocysteine on vascular smooth muscle cells, together with its inhibitory effect on endothelial cell growth, represents an important mechanism to explain homocysteine-induced atherosclerosis.

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