Increased Transforming Growth Factor-Beta$_1$ Circulating Levels and Production in Human Monocytes After 3-Hydroxy-3-Methyl-Glutaryl-Coenzyme A Reductase Inhibition With Pravastatin

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OBJECTIVES We sought to determine whether inhibition of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase with pravastatin affects transforming growth factor-beta$_1$ (TGF-beta$_1$) circulating levels and its production in the monocytes of hypercholesterolemic patients.

BACKGROUND Transforming growth factor-beta$_1$ is a multifunctional growth factor/cytokine involved in many physiologic and pathologic processes, such as vascular remodeling and atherogenesis. Statins have been reported to have a modulatory role in cytokine expression in the monocytes of hyperlipidemic patients.

METHODS We evaluated, in a cross-over study design, plasma TGF-beta$_1$ levels and ex vivo TGF-beta$_1$ production in the monocytes of hypercholesterolemic patients before and after four to six weeks of lipid-lowering treatment with diet or diet plus 40 mg/day of pravastatin. In addition, isolated blood monocytes were subjected to pravastatin treatment and evaluated for TGF-beta$_1$ messenger ribonucleic acid (mRNA) expression and TGF-beta$_1$ in vitro production.

RESULTS Lipid-lowering treatment significantly decreased total cholesterol and low-density lipoprotein cholesterol plasma levels. Pravastatin, but not a low lipid diet, induced a significant increase in TGF-beta$_1$ plasma levels (from 1.7 ± 0.5 ng/ml to 3.1 ± 1.1 ng/ml, $p < 0.001$) and in ex vivo monocyte production (from 1.8 ± 0.8 ng/ml to 3.9 ± 1.0 ng/ml, $p < 0.001$). The increase in TGF-beta$_1$ levels was not related to the changes in the lipid profile observed with pravastatin. An increase of approximately twofold in TGF-beta$_1$ production and in mRNA expression was also observed after in vitro treatment of human monocytes with pravastatin ($5 \mu M$). Co-incubation with mevalonate reversed the in vitro effect of pravastatin.

CONCLUSIONS 3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibition with pravastatin increases TGF-beta$_1$ plasma levels, as well as monocyte production, in hypercholesterolemic patients. The mevalonate pathway plays a role in the regulation of TGF-beta$_1$ expression in human monocytes. A possible implication in the biologic and clinical effects of statins can be suggested.   (J Am Coll Cardiol 2002;39:1752–7) © 2002 by the American College of Cardiology Foundation

Transforming growth factor-beta (TGF-beta) is a multifunctional growth factor peptide reported to be involved in many physiologic and pathologic processes, such as vascular remodeling and atherogenesis (1–5). Virtually every cell in the body, including epithelial, endothelial, hematopoietic and connective tissue cells, produces TGF-beta and has receptors for it (1,2). Recent studies support the role of TGF-beta in the development of human atherosclerotic lesions (6–11) and in the relationship between atherosclerosis, coagulation and fibrinolysis (12). Physiologic levels of TGF-beta$_1$ have been measured in the plasma of normal human subjects, and a possible endocrine role for this peptide has been suggested (13–15). Conflicting results have been found regarding circulating levels of TGF-beta$_1$ in patients with atherosclerotic lesions. Grainger et al. (16) found a severely depressed serum concentration of TGF-beta$_1$ in patients with advanced atherosclerotic lesions, as compared with patients with normal coronary arteries. In contrast, other studies have shown increased levels of TGF-beta, with both the occurrence and severity of atherosclerotic disease (17,18). 3-Hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors are widely used to suppress plasma low-density lipoprotein (LDL) cholesterol levels in patients with primary hypercholesterolemia and have demonstrated a benefit in the progression of atherosclerosis and in the control of cardiovascular events (19). 3-Hydroxy-3-methyl-glutaryl-coenzyme A reductase inhibitors have been used to establish the functional involvement of the mevalonate pathway in the regulation of vascular smooth muscle cell proliferation (20), endothelial function (21), leukocyte-endothelial cell adhesion (22) and mononuclear cytokine production (23,24). Although there have been many products elaborated by monocytes and macrophages, no data have been found, at this time, on the effect of HMG-CoA reductase inhibitors on TGF-beta$_1$, despite the fact this cytokine is synthesized by these cells, which are within the major cellular sources of TGF-beta$_1$ (1,2).

Abbreviations and Acronyms

AHA	=	American Heart Association
DNA	=	deoxyribonucleic acid
HDL	=	high-density lipoprotein
HMG-CoA	=	3-hydroxy-3-methyl-glutaryl coenzyme A
LDL	=	low-density lipoprotein
mRNA	=	messenger ribonucleic acid
TGF-beta$_1$	=	transforming growth factor-beta$_1$

In this study, we conducted a four- to six-week cross-over study of pravastatin (40 mg/day) and a lipid-lowering diet. Before and at the end of treatment, the lipid profile and TGF-beta$_1$ plasma levels were measured. In addition, we evaluated the effect of lipid-lowering treatment on TGF-beta$_1$ production and messenger ribonucleic acid (mRNA) expression in human monocytes cultures. Our results show that four to six weeks of pravastatin treatment significantly increased TGF-beta$_1$ circulating levels and its production in the monocytes of hypercholesterolemic patients. Furthermore, pravastatin induced an increase in mRNA expression and a dose-dependent increase in TGF-beta$_1$ production in in vitro cultures of human monocytes. It would appear, from our observations, that pravastatin has a modulatory role on the TGF-beta$_1$ profile.

METHODS

Subjects and study design. A total of 18 patients were enrolled from the Department of Internal Medicine at the University of Chieti, Italy. Patients with primary hyperlipidemia, with no previous history of ischemic heart disease, were selected. Patients with secondary hyperlipidemia (i.e., hypothyroidism, nephrotic syndrome, diabetes) were excluded. Noncardiovascular conditions associated with a possible pathogenetic role for TGF-beta (i.e., malignancy, acute and chronic liver disease, connective tissue disease) were excluded after a complete medical and laboratory examination.

The study had a randomized, cross-over design. Hypercholesterolemic patients were randomly allocated to receive a low-lipid dietary regimen (American Heart Association [AHA] phase 1 diet) (25) or diet plus pravastatin (40 mg) for four to six weeks, followed by four to six weeks of cross-over treatment with a three-week washout period between regimens. Follow-up clinic visits were scheduled every four to six weeks to monitor biochemical measures. The study was approved by the Ethics Committee of Chieti University, and all patients gave written, informed consent. Serum total cholesterol, triglyceride and high-density lipoprotein (HDL) cholesterol concentrations were determined by conventional enzymatic methods, whereas LDL cholesterol was calculated by using the Friedewald formula (26).

TGF-beta$_1$ plasma assay. Drawing blood and preparing plasma were designed to minimize platelet degranulation (27). A TGF-beta$_1$ specific enzyme-linked immunosorbent assay (R & D Systems Europe, Ltd., Abington, UK) was then used to quantify TGF-beta$_1$ according to the manufacturer's instructions. This assay detects all forms of TGF-beta$_1$, which can be activated by treatment with acid and urea, including lipoprotein-associated TGF-beta$_1$ (28). The sensitivity of this assay was 32 pg/ml; the intra-assay and inter-assay coefficient of variation were 5.3% and 8.8%, respectively.

Mink-lung epithelial cell deoxyribonucleic acid (DNA) synthesis bioassay. Plasma levels and monocyte production of TGF-beta$_1$ were also quantified by using an assay measuring the inhibition of the growth of CCL-64 mink-lung epithelial cells (ATCC, Rockville, Maryland), as previously described (29). Briefly, CCL-64 cells were plated in Dulbecco's modified Eagle's medium (4×10^4 cells/ml), and aliquots of acid-activated plasma samples, a conditioned medium of cultures of peripheral blood monocytes and standards of known concentrations of purified platelet human TGF-beta$_1$ were added in triplicate to the monolayers. Synthesis of DNA was determined by pulsing with 0.5 μCi of ^{3}H-thymidine (2 μCi/ml; Amersham Pharmacia Biotech, Cologno Monzese, Michigan) for an additional 4 h. The TGF-beta$_1$ concentration was determined at a linear portion of the standard curve.

Isolation of blood monocytes and cell cultures and TGF-beta$_1$ expression and production. Peripheral blood monocytes of hypercholesterolemic patients were isolated, and TGF-beta$_1$ mRNA and the protein secretion rate from these cells were studied. Peripheral heparinized blood was diluted 1:1 with normal saline, and mononuclear cells were separated by the Ficoll-Hypaque gradient, as previously described (29). Mononuclear cells recovered at the interface were seeded into Petri dishes, and then adherent monocytes (1×10^6 cells/ml) were incubated at 37°C in a 5% CO_2-humidified atmosphere for 24 h in Roswell Park Memorial Institute with 1% fetal calf serum without and in the presence of pravastatin alone (1 to 10 μM) and pravastatin plus mevalonate (100 μM). After an incubation time of 24 h, monocyte supernatants were harvested and kept at −20°C until use.

Northern blot analysis. Ribonucleic acid was purified from cultured adherent monocytes by a modification of the guanidine hydrochloride extraction method (30). Total RNA was fractionated on 1% formaldehyde agarose gel, transferred to nylon membranes and hybridized according to the standard procedure. The probe used was a human TGF-beta$_1$ isoform (gift of Dr. M. L. McGeidy, Laboratory of Tumor Immunology and Biology, National Cancer Institute, Bethesda, Maryland). The size of the transcript was indicated as relative to 18S and 28S ribosomal RNA, which were assumed to be 1.8 kb and 5.4 kb, respectively.

A densitometer (Ultrascan XL, Pharmacia, Uppsala, Sweden) was used for normalization. The autoradiograms were scanned, and peak areas were measured for relative mRNA levels (TGF-beta$_1$ mRNA/glyceraldehyde-3-phosphate dehydrogenase) in the samples tested.

Table 1. Effect of Lipid-Lowering Treatment on Lipid Profile

|		Randomized Treatment	
	Baseline	**Diet**	**Diet Plus Pravastatin**
Total cholesterol (mg/dl)	263 ± 21	234 ± 22*	206 ± 17†
LDL cholesterol (mg/dl)	175 ± 25	155 ± 18*	127 ± 25†‡
HDL cholesterol (mg/dl)	52 ± 11	52 ± 11	55 ± 12
Triglycerides (mg/dl)	149 ± 38	148 ± 43	129 ± 25

*p < 0.01 and †p < 0.001 compared with baseline. ‡p < 0.01 for pravastatin versus diet treatment. Data are expressed as the mean value ± SD.
  HDL and LDL = high- and low-density lipoprotein, respectively.

Statistical analysis. After testing the data for normality, we used the Student paired *t* test to compare biochemical values at baseline and after each treatment. The baseline value after the washout period was not different from the first baseline value, and no carryover effect was present after each treatment. Multivariate analysis of variance adjusted for age, gender and changes in lipids as co-variates was performed to evaluate TGF-beta$_1$ changes after treatment. The correlation between TGF-beta$_1$ plasma levels and other biochemical variables was assessed by the Pearson correlation test. A comparison between ex vivo TGF-beta$_1$ production in monocytes was made using the nonparametric Mann-Whitney *U* test. All in vitro experiments were conducted in triplicate, with individual preparations of monocytes. Comparisons among in vitro treatment conditions were carried out by analysis of variance, followed by Dunnett's test. The results are expressed as the mean value ± SD. Statistical significance was set at p < 0.05. All computations were carried out using the SAS statistical package (SAS Institute, Cary, North Carolina) (31).

RESULTS

The salient characteristics of the participants in this study are as follows: age, 59 ± 11 years; male/female ratio, 7/11; and body mass index, 24.5 ± 3 kg/m^2. As shown in the Table 1, there was a significant reduction in total and LDL cholesterol with diet and diet plus pravastatin treatment. There were no significant effects on triglyceride and HDL cholesterol levels. As shown in Figure 1, pravastatin treatment resulted in a significant increase in TGF-beta$_1$ levels (1.7 ± 0.5 ng/ml to 3.1 ± 1.1 ng/ml, p < 0.001). There were no significant differences in the mean values of TGF-beta$_1$ from baseline to the end of treatment in the patients treated only with diet (1.7 ± 0.5 ng/ml to 1.6 ± 0.7 ng/ml, p = 0.8). The TGF-beta$_1$–dependent biologic activity was also tested using the mink-lung cells growth inhibition bioassay, which showed that plasma samples from hypercholesterolemic patients inhibited DNA synthesis to 48 ± 8% of the control values (CCL-64 cells in the conditioned medium) (6,400 to 3,100 cpm/well, which was 1.8 ± 0.8 ng/ml on the standard curve); the plasma samples of patients treated with pravastatin showed an increased TGF-beta$_1$–dependent inhibitory activity, which came close to 11.0 ± 0.9% of the control values (6,400 to 700
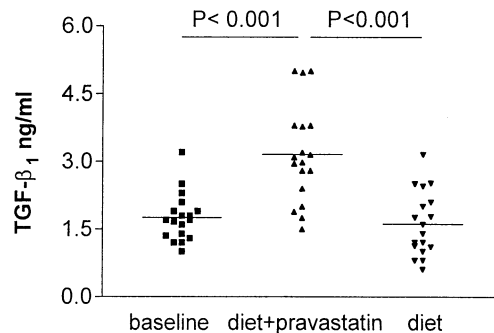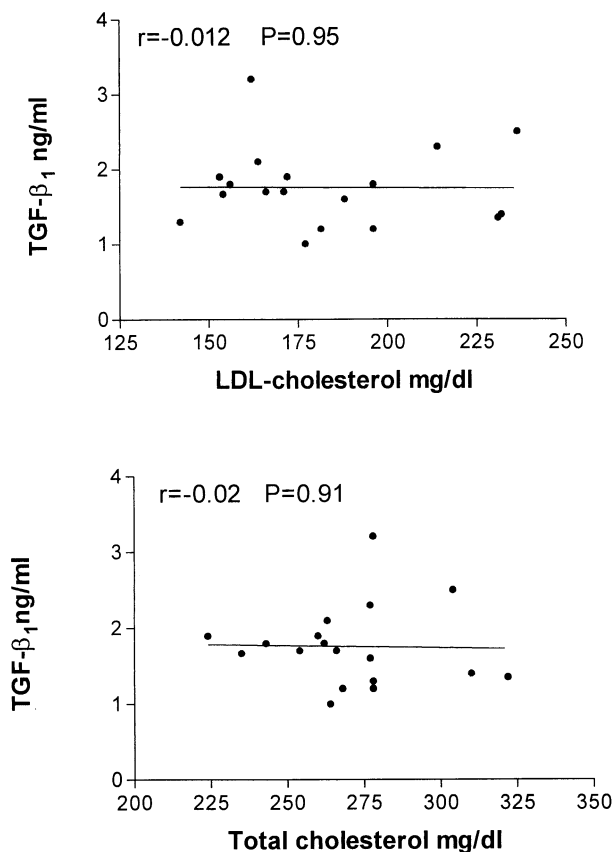

Figure 1. Scatterplot showing transforming growth factor-beta$_1$ (TGF-beta$_1$) plasma levels in hypercholesterolemic patients (n = 18) before treatment (baseline) and after diet plus pravastatin (40 mg/day) or diet alone for four to six weeks.

cpm/well, which was 3.9 ± 1.0 ng/ml on the standard curve).

Univariate analysis showed, at baseline, no significant correlation between TGF-beta$_1$ levels, total cholesterol and LDL cholesterol (Fig. 2). No significant association was also found between TGF-beta$_1$ levels, triglyceride levels (r = −0.31, p = 0.19) and HDL cholesterol levels (r = −0.17, p = 0.47).

On multivariate analysis of variance, with age, gender and

Figure 2. Scatterplots showing the relationships between transforming growth factor-beta$_1$ (TGF-beta$_1$) plasma levels and low-density lipoprotein (LDL) **(top)** and total cholesterol **(bottom)** levels in hypercholesterolemic patients (n = 18).
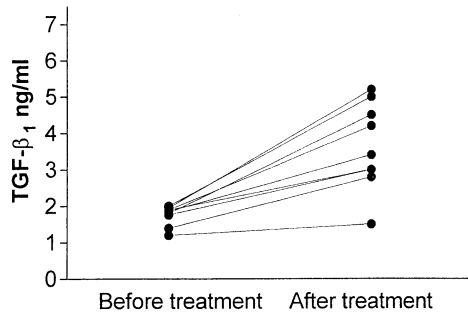
Figure 3. Production of transforming growth factor-beta$_1$ (TGF-beta$_1$) in the monocytes of hypercholesterolemic patients (n = 9) at baseline and after four to six weeks of diet plus pravastatin (40 mg/day) treatment. A conditioned medium of unstimulated cultures was collected after 24 h, and the biologic activity of TGF-beta$_1$ was evaluated after acidification for mink-lung epithelial cell bioassay. Mink-lung cells respond to TGF-beta$_1$ released in the culture medium, with a decrease in deoxyribonucleic acid synthesis, as evaluated by ^{3}H-thymidine incorporation. A comparison with the standard curve and multiplication by the dilution factor (1:10) yielded TGF-beta$_1$ concentration (ng/ml) in the conditioned medium of monocyte cultures.

the magnitude of change in total cholesterol, LDL cholesterol, triglyceride and HDL cholesterol levels as co-variates, TGF-beta$_1$ levels were still significantly increased by pravastatin (p < 0.0001), but not by diet (p = 0.50; difference between therapies: p = 0.0006).

In the total cohort, no significant correlation was observed between the magnitude of changes in TGF-beta$_1$ levels and the magnitude of changes in total and LDL cholesterol after pravastatin treatment (r = 0.18, p = 0.45 and r = 0.87, p = 0.72, respectively), and this confirms that the increase in TGF-beta$_1$ levels after pravastatin therapy was independent of lipids changes.

Moreover, in a subgroup of nine patients, we evaluated whether four to six weeks of lipid-lowering treatment with diet and pravastatin was also effective on TGF-beta$_1$ production in the monocytes of hypercholesterolemic patients. Figure 3 reports, for each patient, an individual change in TGF-beta$_1$ production in the monocytes of hypercholesterolemic patients before and after lipid-lowering treatment, as evaluated by the mink-lung cells growth inhibition bioassay. We found a 2.4-fold mean increase in TGF-beta$_1$–dependent biologic activity after pravastatin treatment, as compared with the baseline values (at baseline: ^{3}H-thymidine incorporation: 4,400 ± 500 cpm/well, which was 1.6 ± 0.2 ng/ml on the standard curve; after pravastatin: 1,790 ± 400 cpm/well, which was 3.8 ± 0.9 ng/ml on the standard curve; p < 0.01).

To elucidate the possible mechanisms for changes in monocyte TGF-beta$_1$ production, we studied the effect of pravastatin on in vitro TGF-beta$_1$ production in monocytes and its mRNA expression. Treatment with pravastatin dose-dependently increases TGF-beta$_1$ production from 4.5 ± 1.0 to 9.5 ± 3 ng/ml at 5 μM (p < 0.01, n = 5). No further significant increase was observed at 10 μM. Co-

incubation of pravastatin (5 μM) with mevalonate (100 μM) reversed the increase in TGF-beta$_1$ production induced by pravastatin (Fig. 4A). Figure 4B is a representative Northern blot analysis of TGF-beta$_1$ mRNA expression from human monocyte cultures with or without pravastatin (5 μM). Significant pravastatin-induced TGF-beta$_1$ mRNA expression was observed after 24 h of incubation time; this increase was prevented by 100 μM of mevalonate. A 2.3-fold mean increase was found after densitometric analysis of three consecutive experiments.

DISCUSSION

Lipid-lowering therapy for cardiovascular disease is common, and all of the mechanisms responsible for the benefit of statins remain to be defined (19). Our study focused on the impact of statin therapy on the levels of TGF-beta$_1$, a growth factor/cytokine implicated in the regulation of chronic inflammation and atherogenesis (2). Our study shows that four to six weeks of lipid-lowering treatment with pravastatin (40 mg/day) significantly increased TGF-beta$_1$ circulating levels, as compared with lipid-lowering treatment with the AHA phase 1 diet. Ex vivo monocyte production of TGF-beta$_1$ was also increased after pravastatin treatment. Finally, pravastatin induced a dose-dependent increase in TGF-beta$_1$ production and a significant increase in mRNA expression in in vitro cultures of human monocytes. Thus, taken together, our observations demonstrate a pravastatin-dependent upregulation of the TGF-beta$_1$ profile.

Comparison with previous studies. Knowledge of the effect of statin treatment on TGF-beta$_1$ expression is limited. Contrasting results have been found in TGF-beta$_1$ tissue expression after HMG-CoA reductase inhibitor treatment. Kitahara et al. (32) demonstrated that HMG-CoA reductase inhibition, which was able to suppress balloon injury-induced neointimal thickening, was associated with an increase in vascular TGF-beta expression. In contrast, other studies demonstrated that statins were able to inhibit TGF-beta$_1$ expression in diabetic rat glomeruli and cultured rat mesangial cells (33,34). Park and Galper (35) recently demonstrated, in embryonic chicken atrial cells, in the absence of lipoproteins, that inhibition of the cholesterol pathway by HMG-CoA reductase inhibitors resulted in a coordinated upregulation of the expression of TGF-beta$_1$, its type II receptor and plasminogen activator inhibitor-1 promoter activity (35). These findings seem to be cell-type specific and associated with different in vitro and animal experimental conditions.

Our data extend these findings by demonstrating the effect of a HMG-CoA reductase inhibition on TGF-beta$_1$ levels in hypercholesterolemic patients and in in vitro human monocytes.

According to several other studies, the beneficial effects of statins may not be related to their cholesterol-lowering actions (36). We found that the magnitude of increase in
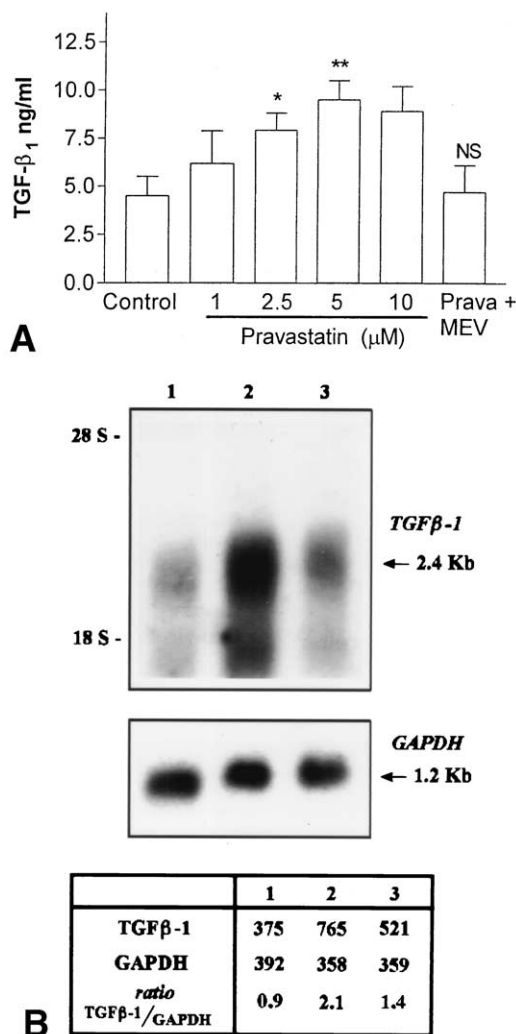
Figure 4. The effect of pravastatin (Prava) and mevalonate (MEV) on the production and messenger ribonucleic acid (mRNA) levels of transforming growth factor-beta₁ (TGF-beta₁). **(A)** Bioassay of TGF-beta₁ activity in a conditioned medium of monocyte cultures incubated with pravastatin (1–10 μM) or pravastatin (5 μM) plus mevalonate (100 μM). A conditioned medium from the cultures was collected after 24 h and acidified for mink-lung epithelial cell bioassay. Mink-lung cells respond to TGF-beta₁ released in the culture medium, with a dose-dependent decrease in deoxyribonucleic acid (DNA) synthesis, as evaluated by ³H-thymidine incorporation. A comparison with the standard curve and multiplication by the dilution factor (1:10) yielded a dose-dependent increase in the TGF-beta₁ concentration (ng/ml) in the conditioned medium of monocyte cultures. Mevalonate reversed the TGF-beta₁–dependent effect on mink-lung cells. The results are expressed as the mean value ± SD of three independent experiments performed in triplicate. Comparisons among treatment conditions were carried out by analysis of variance, followed by Dunnett's test. *p < 0.05, **p < 0.01 and p = NS versus untreated (control) cells. **(B)** Representative Northern blot analysis of TGF-beta₁ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in human peripheral blood monocytes after incubation with pravastatin (5 μM) alone or mevalonate (100 μM) together with pravastatin (5 μM). Mononuclear cells were cultured 24 h in serum-free conditions. Total RNA (5 μg per lane) was electrophoresed on 1.5% formaldehyde agarose gels, transferred to nitrocellulose and hybridized with ³²P random primer-labeled TGF-beta₁ complementary DNA. The sizes of the transcripts were determined by comparison with 28S and 18S ribosomal RNA. Molecular size markers are shown on the left. **Lane 1** = untreated cells; **lane 2** = pravastatin-treated cells; and **lane 3** = cell treated with pravastatin plus mevalonate. Quantitative data obtained from imaging are expressed in arbitrary units.

TGF-beta₁ circulating levels observed after pravastatin did not show any correlation with the magnitude of change in total and LDL cholesterol levels. Furthermore, after adjustment for age, gender and changes of lipid levels, TGF-beta₁ concentrations were still significantly increased by pravastatin, but not by diet alone.

HMG-CoA reductase inhibition and TGF-beta₁. The involvement of the melavonate pathway in the upregulation of TGF-beta₁ expression in human monocytes was demonstrated by prevention of the stimulatory effect caused by pravastatin, when mevalonate was simultaneously added to pravastatin in the culture medium. We did not undertake further studies to evaluate different branches of the cholesterol metabolic pathway (37); however, previous studies demonstrated that HMG-CoA reductase inhibitors may upregulate TGF-beta signaling through a geranylgeranylation pathway (35).

The finding that HMG-CoA reductase inhibition is able to induce an increase in the monocyte expression of TGF-beta₁ could have important implications for the mechanism of action of these agents. TGF-beta₁ showed a wide range of activities on vascular and inflammatory cells, and may have different functions during various stages of atherosclerosis. In vitro and in vivo experimental studies demonstrated a role of TGF-beta₁ in vascular smooth muscle cell proliferation, extracellular matrix synthesis and myointimal hyperplasia (38–41).

Recent studies demonstrated, in mice heterozygous for the deletion of the TGF-beta₁ gene, subjected to a cholesterol-enriched diet, a significant inflammatory response in the vascular wall, as compared with normal mice, suggesting a TGF-beta₁–dependent anti-inflammatory role in the pathogenesis of vascular lipid inflammatory lesions (42). In addition, in vitro studies showed a role of TGF-beta₁ in the control of LDL receptor expression on human liver and vascular smooth muscle cells (43,44). Our data cannot suggest any pathophysiologic implications for TGF-beta₁ upregulation induced by pravastatin. However, because some studies reported depressed, active TGF-beta₁ levels among patients with advanced atherosclerosis (16,18), and others demonstrated that a genetically induced deficiency of TGF-beta₁ in mice promotes atherosclerosis (42), a possible beneficial contribution of the statin-dependent TGF-beta₁ increase could be suggested.

Conclusions. An increase in TGF-beta₁ plasma levels and monocyte production by lipid-lowering treatment with an HMG-CoA reductase inhibitor is a novel biologic effect of these compounds, which should be kept in mind to explain their pharmacologic action. Our results seem to show that the increase in TGF-beta₁ is due to interference with the mevalonic pathway, not related to a cholesterol-lowering effect. Additional studies are necessary to clarify the pathophysiologic consequences of TGF-beta pathway activation after pravastatin treatment.

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