REVERSAL OF STATIN TOXICITY TO HUMAN LYMPHOCYTES IN TISSUE CULTURE

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SUMMARY

Hydroxymethylglutaryl-CoA reductase inhibitors (statins) are widely used to inhibit biosynthesis of cholesterol in individuals with elevated serum levels of this risk factor for cardiovascular disease. We find that statins are toxic to human lymphocytes in cell culture at concentrations less than 0.1 μg/ml. Addition of their own plasma reverses this toxicity in some, but not all, individuals. Addition of coenzyme Q_{10} (CoQ_{10}) with plasma is more effective than plasma alone in reversing toxicity in some individuals. Apparently, two factors are required to reverse the cellular toxicity of statins: CoQ_{10} and a plasma factor found in a subset of individuals. These observations may provide the basis for a method to assess individual susceptibility to statin toxicity and to predict which individuals may benefit from supplements of CoQ_{10}.

KEY WORDS

coenzyme Q_{10}, ubiquinone, hydroxymethylglutaryl-CoA reductase inhibitors, lymphocytes, cultured cells

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INTRODUCTION

A previous report /1/ described the growth of human lymphocytes in tissue culture as a means to assess metabolic and nutritional status. This technique has been used to explore the sparing effect of asparagine on glutamine requirements of lymphocytes /2/ and to study the effect of sulfite on their cysteine requirement /3/. In addition to examining the functioning of specific metabolic pathways, this technique can be used to study the biochemical effects of various substances on the growth of human cells.

Hydroxymethylglutaryl-CoA reductase inhibitors (statins) inhibit an enzyme in the early rate-limiting step in cholesterol biosynthesis. These compounds are frequently prescribed for patients with high serum cholesterol levels /4-6/. Previous reports have indicated that treatment with statins lowers plasma levels of coenzyme Q_{10} (CoQ_{10}), with potential deleterious effects /7/. Oral supplements of CoQ_{10} preserve CoQ_{10} levels in both plasma and platelets /8/. Statins also reduce lymphocyte proliferation *in vitro* /9/. Because these drugs are commonly prescribed, experiments were designed to study statin inhibition of lymphocyte growth and to explore possible mechanisms to reverse this inhibition.

MATERIALS AND METHODS

Cell culture methods

A chemically defined, minimal growth medium /1/ was modified to contain 5-fold increased amino acid concentrations except for cysteine and glutamine. This increase was necessary because some cells proliferated at such high levels that the minimal growth medium became prematurely depleted of most amino acids. Lymphocytes were added to the growth medium (alone and with other additions) at a concentration of 1×10^5 cell/ml. This solution was added to 96-well microtiter plates, 0.2 ml per well, and incubated for 4 days at 37.5°C in humid air with 5% CO_2. The cells were then pulsed with tritium-labeled thymidine (methyl-H^3, 5.9 μM, specific activity = 1.0 mCi/μM, ICN Biomedicals, Costa Mesa, CA). After 24 h further incubation, cells were harvested with an Inotech Cell Harvester (Inotech Biosystems International, Rockville, MD). The harvest filters

were dried and their tritium activity measured on a Packard Matrix 96 Beta Counter (PerkinElmer Life Sciences, Boston, MA). Lymphocyte proliferation was determined from the mean activity of three identical wells.

Some test wells also included plasma, CoQ_{10}, or both. Plasma was obtained during lymphocyte separation from the same subject's blood sample. It was diluted and added to the selected wells at a final concentration of 0.1%.

CoQ_{10} (Sigma, St. Louis, MO) was dissolved in ethanol and then diluted and added to the wells at a final concentration of 0.5 μg/ml. An equivalent amount of ethanol added to control samples showed no effect on cell growth.

Preparation of lymphocytes

Peripheral blood samples were obtained from 323 subjects by phlebotomy into tubes containing sodium heparin. The lymphocytes were isolated by the method of Boyum /10/. They were washed twice and resuspended in a medium containing the buffer and minerals of the growth medium, but not the glucose, amino acids or other organic constituents, which were added later. The resuspended cells were counted by Coulter Counter (model ZBI) and diluted to 2×10^6 cells/ml.

Statin stock solutions

Mevinolin (lovastatin from *Aspergillus* sp., minimum 98% by HPLC; Sigma, St. Louis, MO) was dissolved in ethanol, 1 mg/ml. Stock solutions of other statins were prepared by thoroughly grinding and dissolving commercial tablets, 10-mg dose, in 10 ml of ethanol and centrifuging the solution to remove insoluble debris (atorvastatin from Lipitor® tablet, lovastatin from Mevacor® tablet, pravastatin from Pravachol® tablet and simvastatin from Zocor® tablet). The resulting statin concentrations were assumed to be 1 mg/ml (not measured). These statins are known to be at least this soluble in ethanol. About 10 minutes dissolving time was allowed for the ground tablets, and cell culture results were the same for pure lovastatin (mevinolin) and lovastatin from a ground tablet with insoluble excipients. The stock concentrations of 1 mg/ml correspond to 1.65 μmole/ml for atorvastin, 2.47 μmole/ml for lovastatin, 2.24 μmole/ml

for pravastatin, and 2.39 μmole/ml for simvastatin (relative values 1.00, 1.49, 1.35 and 1.44, respectively). These stock solutions were diluted in the growth medium to add a wide range of concentrations of statins to the lymphocyte cell cultures.

This study was approved by our institutional review board, and subjects signed informed consent forms.

RESULTS AND DISCUSSION

All statins tested were toxic to lymphocytes at concentrations less than 0.1 μg/ml, for all subjects (Fig. 1, Table 1). Maximum blood concentrations of these statins are about 0.01 to 0.07 μg/ml at an oral dose of 40 mg/day /11/. Under our conditions, toxicity to lymphocytes was detectable for all tested statins at 0.03 μg/ml, but we used higher levels that more easily show the effects of added CoQ_{10} and plasma. We know of no data comparing the relative statin sensitivity of lymphocytes and other human cells or about possible differences between *in vivo* and *in vitro* conditions.

Pure mevinolin showed effects on cell growth that were indistinguishable from the same concentration of lovastatin and other statins, based on results from at least four subjects for each statin.

TABLE 1

Inhibition of lymphocyte proliferation by 0.1 μg/ml atorvastatin and reversal by plasma and CoQ_{10}

Subjects tested	323	100%
Inhibited >80%	124	38%
Inhibited 60-80%	174	54%
Inhibition >80% reversed by 0.1% plasma	125	39%
Inhibition 60-81% reversed by 0.1% plasma	80	25%
Reversal improved >20% by 0.1% plasma + 0.5 μg/ml CoQ_{10}	74	23%
Reversal improved <20% by 0.1% plasma + 0.5 μg/ml CoQ_{10}	42	13%

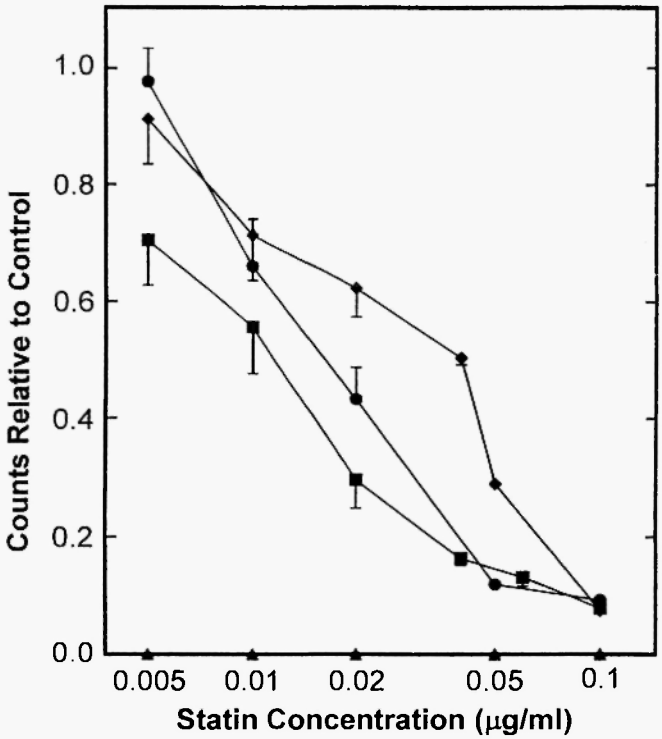

Fig. 1: Inhibition of lymphocyte proliferation by mevinolin (squares), atorvastatin (diamonds) and pravastatin (circles) in different subjects. Cell proliferation was measured by counting incorporation of tritiated thymidine (mean of triplicate samples, with standard deviation). Equal statin mass concentrations correspond to relative molar concentrations of 1.49 for mevinolin, 1 for atorvastatin and 1.35 for pravastatin.

In some subjects, addition of 0.1% of the subject's own plasma to the medium reversed the toxicity at high levels of statin (Fig. 2). However, this property was not universal (Fig. 3, circles). Indeed, at some levels, plasma enhanced statin toxicity. In some subjects whose own plasma was ineffective, toxicity could be mitigated by other plasma that was known to be effective (Fig. 3, triangles). Table 1 summarizes the results in 323 subjects.

Because many individuals have taken these drugs without experiencing obvious side effects, humans evidently have some

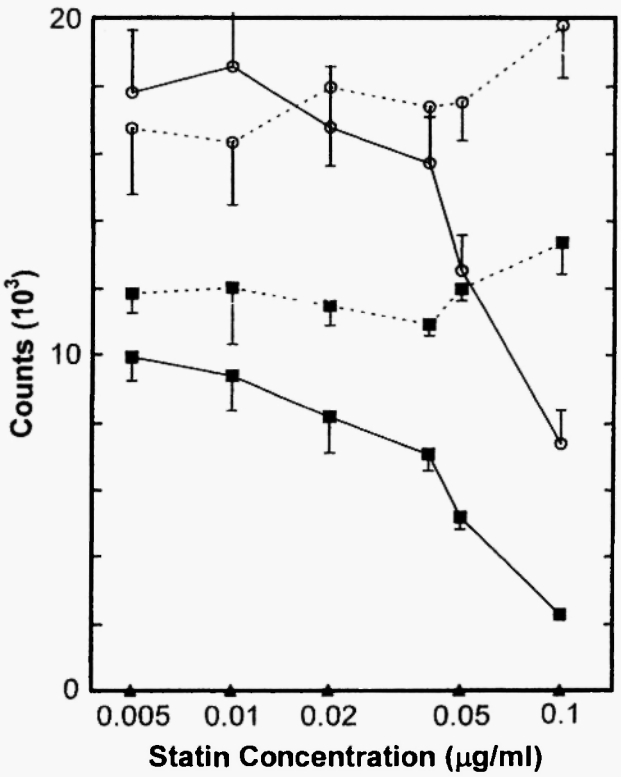

Fig. 2: Reversal of atorvastatin toxicity by subjects' own plasma. Squares and circles represent two different subjects; solid lines connect points for statin alone and dashed lines connect points with 0.1% plasma.

mechanism for modulating the observed toxicity. Statins are designed to inhibit synthesis of mevalonic acid, a precursor of cholesterol, which effectively suppresses cholesterol biosynthesis *in vivo*. However, mevalonic acid is also the precursor of a family of isoprenoid compounds, some of which serve essential functions in the body. One such compound is CoQ_{10} (ubiquinone), which is required for electron transport in mitochondria. Because statins are known to lower CoQ_{10} levels, CoQ_{10} was added to the medium containing atorvastatin to determine its effects on the observed toxicity. CoQ_{10} stimulated cell growth in some subjects, but did not effectively reverse statin toxicity when added alone to the culture medium (Fig. 4, diamonds). However,

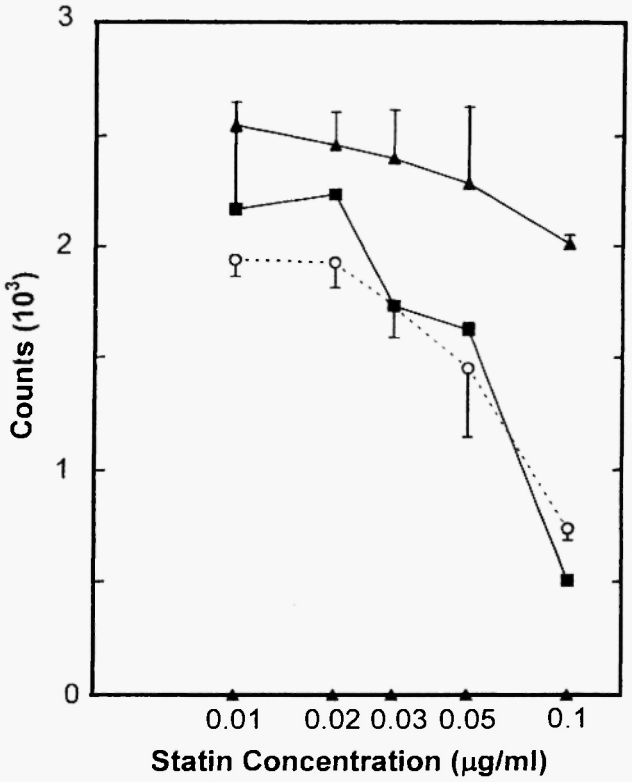

Fig. 3: Reversal of atorvastatin toxicity (squares) by 0.1% active plasma (triangles). This subject's own plasma (circles) did not reverse toxicity. Data were not obtained at a statin concentration of 0.005 μg/ml.

it was effective in some subjects when combined with plasma (Fig. 4, triangles).

Preliminary studies to identify the protective factor in plasma have shown it is heat labile. Heating plasma samples for 5 minutes at 65°C is sufficient to destroy activity. Fractionation of plasma using an Aminco centrifugal filter device (Millipore Corp., Bedford, MA) showed activity only in the fraction with a molecular weight greater than 100,000. Apparently the active component is either a large protein or a small molecule associated with a high molecular weight species.

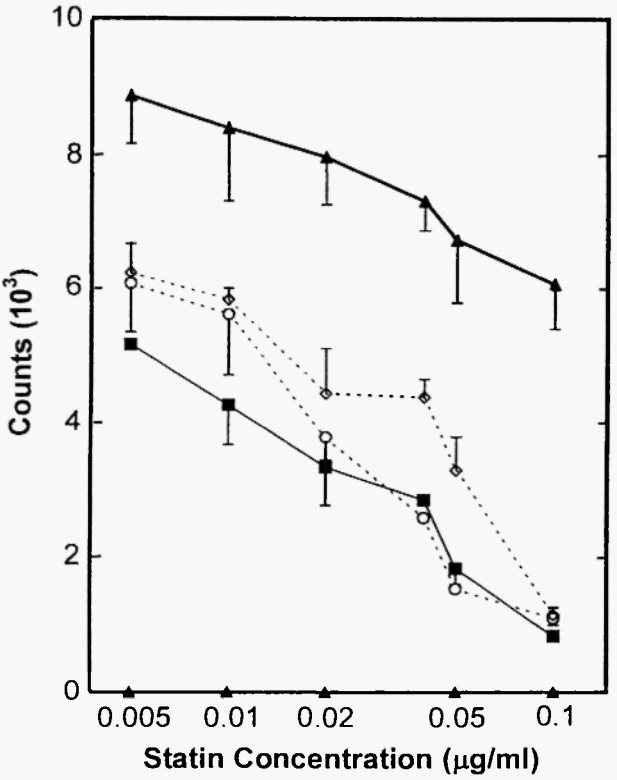

Fig. 4: Reversal of atorvastatin toxicity (squares) by a combination of the subject's own plasma (0.1%) and CoQ_{10} (0.5 μg/ml) (triangles). The plasma alone (circles) and CoQ_{10} alone (diamonds) had little or no protective effect in this subject.

All subjects tested (n = 323) showed sensitivity to the statins at a concentration of 0.1 μg/ml or less. The most sensitive 38% of subjects exhibited >80% inhibition; the next most sensitive 54% was inhibited by more than 60% (Table 1). Based on the ability of plasma and CoQ_{10} to reverse this toxicity, we divided the subjects into three groups. Group 1 subjects had substantial reversal from plasma alone, arbitrarily selected as >60% of control activity (63% of subjects). This reversal by plasma might be mediated by CoQ_{10} and/or other plasma constituents. Except for pravastatin, statins are tightly bound to plasma proteins, most importantly to albumin /11/. We hypothesize that this

group would be able to take statins safely if their CoQ_{10} plasma level remains adequate. Lymphocytes from group 2 recovered significantly more activity (>20% more) when both their own plasma and CoQ_{10} were included in the medium (23% of subjects, Table 1). Subjects in this group seem most likely to benefit from a supplement of CoQ_{10}. Cells from group 3 (13% of subjects) showed <30% of control activity even when a combination of plasma and CoQ_{10} was included in the medium. Apparently, this group does not produce sufficient amounts of the protective plasma factor. We hypothesize that these subjects would be most likely to have adverse reactions to statins.

Lymphocyte culture methods might prove useful to screen candidates for statin treatment, by determining whether the drug will impact lymphocyte function and whether a supplement of CoQ_{10} should be recommended. Further studies are planned to determine the identity of the plasma factor and to establish mechanisms of reversal.

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