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The HMG-CoA reductase inhibitor simvastatin inhibits IFN-γ induced MHC class II expression in human vascular endothelial cells

Statins as a potential novel immunosuppressive agent

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Summary

HMG-CoA reductase inhibitors, or statins, are lipid-lowering agents that have been shown to effectively decrease severe rejection periods and development of transplant coronary artery disease after heart transplantation. Precise regulation of major histocompatibility complex class II (MHC class II) gene expression plays a pivotal role in control of the immune response after transplantation. The aim of this study was to evaluate the potential role of HMG-CoA reductase inhibitors in regulating the immune response. We have examined the effects of simvastatin on MHC class II expression in primary human endothelial cells. Using RNAse protection assay and flow cytometry, we observed that simvastatin dose-dependently reduced interferon-gamma (IFN-γ) induced MHC class II expression (mRNA and protein). In contrast, simvastatin did not affect the expression of MHC class I, pointing to specific actions in the MHC class II signalling cascade. The transcriptional coactivator CIITA is the general regulator of both constitutive and inducible MHC class II expression. After stimulation with IFN-γ, the CIITA gene is selectively activated via one (promoter IV) of its four promoters. Interestingly, mRNA levels of CIITA were decreased after treatment with simvastatin. In addition, using transient transfections of promoter-reporter constructs we observed that the activity of CIITA promoter IV was decreased by simvastatin. In conclusion, simvastatin selectively decreases IFN-γ-induced MHC class II expression in human primary endothelial cells through actions on the CIITA promoter IV. Thus, the beneficial effects of statins reported after heart transplantation may result from this immunosuppressive action, suggesting possible therapeutic use for other immunological disorders as well.

Keywords: MHC class II; statins; human endothelial cells; immunosuppression

Introduction

Cardiac allograft vasculopathy (CAV) is an accelerated form of coronary artery disease responsible for the majority of late deaths after cardiac transplantation [1]. Although the aetiology and pathogenesis of CAV are not yet completely understood, the initiating event that subsequently leads to CAV has been related to endothelial injury [2]. Loss of endothelial monolayer integrity will lead to infiltration of lipoproteins and inflammatory cells (activated lymphocytes) as well as formation and adherence of platelet microthrombi, thus increasing vulnerability of the intima to cellular rejection processes. The damage to the endothelium may be caused by immune mechanisms. Indeed, the presence of major histocompatibility complex class II (MHC class II) antigens on endothelium has been shown to be associated with the development of CAV [3].

MHC class II molecules present exogenous antigenic peptides to CD4+ T lymphocytes. A very tight regulation of MHC-II expression is thus crucial for the control of the immune response. Two

main types of MHC class II expression can be distinguished, constitutive or inducible [4]. MHC class II is constitutively expressed in only a very restricted number of cell types, specialised in antigen presentation, such as dendritic cells and B lymphocytes. MHC class II expression can be induced by interferon-gamma (IFN-γ) in a large variety of other cell types, among which vascular endothelial cells. Expression of MHC class II genes is regulated primarily at the level of transcription, and the class II transactivator CIITA has been found to be a master regulator in this process [5, 6]. CIITA expression patterns correlate with those of MHC class II genes, such that it is constitutively expressed in MHC class II-positive professional antigen presenting cells and seems to be an obligatory mediator of IFN-γ induced MHC class II expression. The CIITA gene is controlled by 4 different promoters [7]. These promoters are activated in a selective manner: two promoters direct constitutive expression in dendritic cells (P-I) and in B lymphocytes (P-III), whereas another promoter (P-IV) mediates IFN-γ inducible expression.

Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (known as statins) consist of a new family of chemically related molecules, selected for their lipid-lowering effect. Statins are extensively used in medical practice and large clinical trials have demonstrated that this class of lipid-lowering drugs greatly reduces cardiovascular-related morbidity and mortality in patients with and without coronary artery disease [8–11]. A few clinical trials have also demonstrated better outcomes for patients after cardiac transplantation under pravastatin or simvastatin therapy [12, 13]. The exact mechanism of the latter's beneficial effect is not known, but recent *in vitro* findings indicate that statins, besides their lipid-lowering effects, have anti-inflammatory properties and thus may regulate important molecules in vascular biology [14]. Indeed, a simvastatin-induced decrease in CAV in a rat heart transplantation study was independent of any change in blood cholesterol level [2]. Knowing the role of endothelial immune activation in the pathogenesis of CAV, we evaluated the effect of simvastatin on IFN-γ induced MHC class II expression in primary human endothelial cells.

Methods

Reagents

Human recombinant IFN-γ was obtained from Endogen (Woburn, MA) and simvastatin (Merck Sharp and Dohme, Whitehouse Station, NJ) was obtained from commercial sources. Mouse anti-human MHC class II and MHC class I fluorescein isothiocyanate-conjugated (FITC) and unconjugated monoclonal antibodies were purchased from Pharmingen (San Diego, CA). L-mevalonate was purchased from Sigma (St. Louis, MO).

Cell isolation and culture

Human vascular endothelial cells (ECs) were isolated from saphenous veins by collagenase treatment (Worthington Biochemicals, Freehold, NJ), and cultured in dishes coated with gelatin (Difco, Liverpool, England) as described elsewhere [15]. Cells were maintained in medium 199 (M199; BioWhittaker, Wokingham, England) supplemented with 100 U/ml penicillin/streptomycin (BioWhittaker), 5% FCS (Gibco, Basel, Switzerland), 100 μg/ml heparin (Sigma) and 50 μg/ml endothelial cell growth factor (Pel-Freez Biological, Rogers, AK). Culture media and FCS contained less than 40 pg LPS/ml as determined by chromogenic Limulus amoebocyte-assay analysis (QLC-1000; BioWhittaker). Endothelial cells were >99% CD31 positive as characterized by flow cytometry and were used at passages 2–4 for all experiments.

T lymphocytes were isolated from freshly prepared human peripheral blood mononuclear cells obtained from leukopacs of healthy donors following Ficoll-Hypaque gradient and subsequent adherence to plastic culture flasks (90 min., 37 °C). Lymphocytes were cultured in RPMI 1640 medium (BioWhittaker) containing 10% FCS.

Flow cytometry

Cells were incubated with FITC-conjugated specific antibody (60 min, 4 °C) and analyzed in a Becton Dickinson FACScan flow cytometer as described [15]. At least 100,000 viable cells were analyzed per condition. Data were analyzed using CELLQUEST software (Becton Dickinson).

RNAse protection assay

Total RNA was prepared with Tri reagent (MRC, Inc., Cincinnati, OH) according to the manufacturer's instructions. RNAse protection assays with 15 μg of RNA per reaction were carried out as described previously [16] using human probes for MHC class II (DR-α), CIITA, and GAPDH as a control for RNA loading. Signal quantitation was determined using a PhosphorImager analysis system (Molecular Dynamics, Sunnyvale, CA). Levels of DR-α and CIITA RNA in any given sample were normalized to the GAPDH signal for that sample.

Reporter gene assay

A CIITA promoter IV-reporter gene plasmid was created by subcloning the CIITA 5'-flanking region, i.e. –461(KpnI)/+75 fragment of exon 1 type IV, upstream of the firefly Luciferase gene of plasmid pGL3-basic (Promega, Madison, WI). Primary human vascular ECs (0.5×10^6) were transiently transfected using 7.2 μl FuGENE transfection reagent (Roche, Indianapolis, IN), 0.3 μg pGL3/461 and 25 ng pRL (Promega) in 150 μl of M199. Eight hours later, cells were rinsed and cultured for an additional 12 hrs under the respective stimulating conditions. Reporter gene expression was measured using the dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Mixed lymphocyte reaction (MLR)

MLRs were performed as described previously [17]. Briefly, human ECs were cultured on 96 well plates pretreated with the respective stimuli and irradiated. Purified allogenic human T lymphocytes were then mixed (2-4×

10^5) with the coated cells for 5 days at 37 °C. Then, cells were pulsed with 10 µCi [^{3}H]thymidine (NEN Life Science Products, Boston, MA) followed by further culture of 24 hrs. Cells were harvested using 0.4 N NaOH and radioactivity was counted by a liquid scintillation analyser (Packard, Downers Grove, IL). Samples were assayed in duplicate.

Results

In order to study the effect of simvastatin on endothelial MHC class II induction by IFN-γ, confluent vascular ECs were cultured in the presence of 500 U/ml IFN-γ in combination with different doses of simvastatin. Surface MHC class II expression was analyzed by flow cytometry after 48 hrs. As can be observed in Fig. 1a, ECs did not express MHC class II under resting conditions and IFN-γ treatment induced expression of this molecule. Simvastatin effectively repressed this induction of MHC class II by IFN-γ in a dose-dependent manner (Fig. 1b). The effect of simvastatin was observed over a range of 0.2–10 µM. Treatment with simvastatin alone had no effect on MHC class II expression (data not shown). HMG-CoA reductase inhibitors, such as simvastatin, block the rate-limiting enzyme in the cholesterol synthesis pathway, preventing the production of L-mevalonate [18]. In the presence of L-mevalonate, the effect of simvastatin on IFN-γ induced MHC class II expression was abolished (Fig. 1c). As shown in Fig. 1d, ECs expressed MHC class I under resting conditions, and IFN-γ treatment further induced the expression of this molecule. Simvastatin, however, appeared unable to inhibit MHC class I expression.

We then verified that the simvastatin-induced inhibition of MHC class II expression was not due to a general toxic effect of the substance, and thus determined cell viability and protein synthesis after treatment with 500 U/ml IFN-γ alone, or in combination with 10 µM simvastatin for 24 hrs. As

Figure 1

Simvastatin decreases IFN-γ induced MHC class II protein expression on human endothelial cells. Flow cytometric analysis for MHC class II (*a-c*) and MHC class I (*d*). *a*, Human vascular endothelial cells (ECs) treated with IFNγ (500 U/ml, 48 hrs) alone (red line), or with simvastatin (10 µM, light blue line). *b*, ECs treated with IFN-γ (500 U/ml, 48 hrs) alone (red line), or with simvastatin 0.2 µM (green line), 2 µM (orange line), or 10 µM (light blue line). *c*, ECs treated with IFN-γ alone (500 U/ml, 48 hrs) (red line), or with simvastatin (10 µM) (light blue line) and with simvastatin and L-mevalonate (500 µM) (green line). *d*, ECs treated with IFN-γ alone (500 U/ml, 48 hrs), or with simvastatin (10 µM, red line). For all panels, solid histograms represent MHC class II (*a-c*) or MHC class I (*d*) expression under unstimulated conditions. Each panel is a histogram representing cell numbers (y axis) vs. log fluorescence intensity (x axis) for 15,000 viable cells. Similar results were obtained in independent experiments with ECs from five different donors.

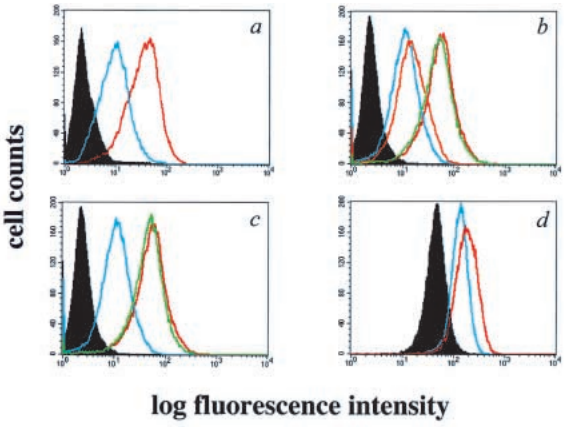


Figure 2

Simvastatin does not affect human endothelial cell viability and protein synthesis. ECs were treated with 500 U/ml IFN-γ (1) for 24 hrs in the presence or absence of 10 µM simvastatin (2). *a*, Cell viability was determined using trypan blue. *b*, Protein synthesis was measured by BCA (Pierce). Results were obtained in independent experiments with ECs from three different donors.

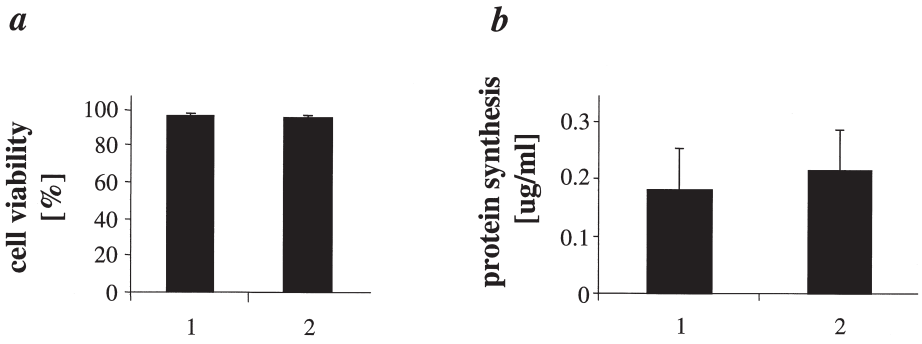
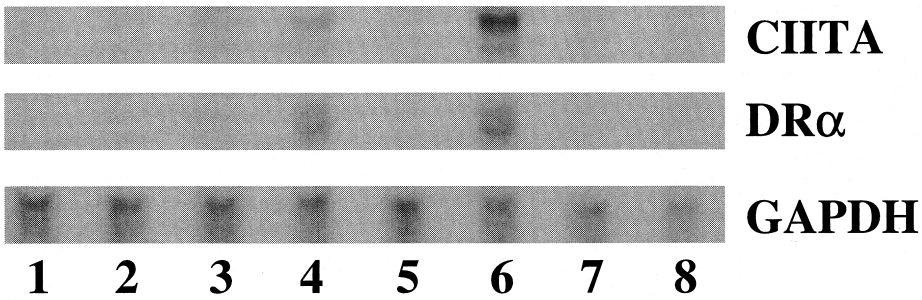


Figure 3

Effect of simvastatin on IFN-γ induced MHC class II expression is mediated by the transactivator CIITA. RNAse protection assay using probes for MHC class II (DR-α), CIITA and GAPDH. Human vascular endothelial cells unstimulated (1), treated with IFN-γ (500 U/ml) alone for 4 hrs (2), 8 hrs (4) or 12 hrs (6), or in combination with simvastatin (10 µM) for 4 hrs (3), 8 hrs (5) or 12 hrs (7), or treated with simvastatin (10 µM) alone for 12 hrs (8). GAPDH was used as a control for RNA loading. Similar results were obtained in independent experiments with ECs from three different donors.



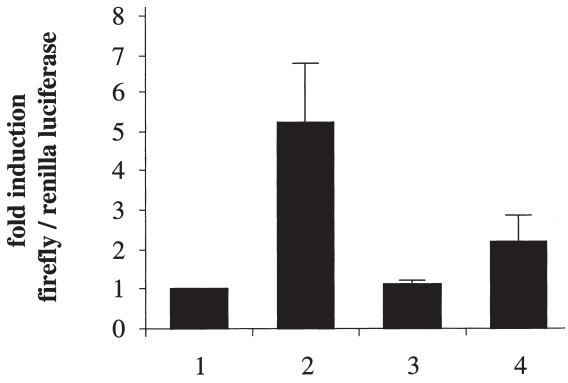


Figure 4

Functional analysis of CIITA promoter IV in response to simvastatin. Plasmid pGL3/461 and a reference plasmid (pRL) were transiently transfected into human vascular ECs. Human ECs unstimulated (1), treated with IFN-γ (500 U/ml; 12 hrs) alone (2), or with simvastatin (10 μM) (3), or with simvastatin (10 μM) and L-mevalonate (500 μM) (4). Results were obtained in independent experiments with ECs from three different donors.

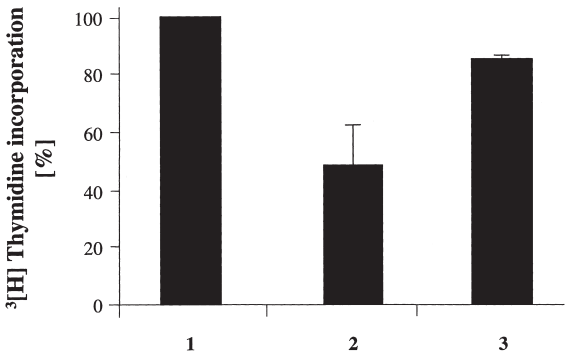


Figure 5

Functional consequences of inhibition of MHC class II antigens by simvastatin on T lymphocyte proliferation. [^{3}H]thymidine incorporation measured in allogenic T lymphocytes exposed (6 days) to human ECs pretreated for 48 hrs with 500 U/ml IFN-γ alone (1) (arbitrarily defined as 100%), or with 10 μM simvastatin (2), or with simvastatin (10 μM) and L-mevalonate (500 μM) (3). Results were obtained in independent experiments with ECs from three different donors.

shown in Figure 2, treatment with simvastatin did not significantly affect cell viability or protein synthesis of human ECs.

To determine the level at which simvastatin exerts its inhibitory action on IFN-γ induced MHC class II expression, we performed RNAse protection assays with probes for MHC class II (DRα) and the class II transactivator CIITA. As expected, human ECs did not express DRα or CIITA mRNA under resting conditions (Fig. 3, lane 1). Both mRNAs, however, were inducible by stimulation with 500 U/ml IFN-γ for 8 or 12 hrs (Fig. 3, lanes 4 and 6). Interestingly, this induction by IFN-γ was completely abrogated by 10 μM simvastatin for both DRα or CIITA (Fig. 3, lanes 5 and 7). Treatment with simvastatin alone did not affect expression levels of either mRNA (Fig. 3, lane 8).

The CIITA gene is controlled by 4 different promoters, one of which (P-IV) mediates IFN-γ inducible expression [7]. In order to test for the functional activity of CIITA promoter IV under various conditions, we have created a construct in which firefly luciferase was placed under the control of this promoter. After transient co-transfection of this promoter-reporter gene construct and a reference plasmid into human ECs, cells were stimulated for 12 hrs, and expression levels were analyzed. As shown in Fig. 4, IFN-γ treatment (500 U/ml) increased the expression of firefly luciferase 5-fold. Simvastatin (10 μM), however, effectively repressed this IFN-γ induced expression to baseline levels. The simvastatin-induced repression was partly inhibited by L-mevalonate.

To investigate the functional consequences of simvastatin-induced inhibition of MHC class II expression, we performed mixed lymphocyte reactions. Allogenic T lymphocytes were thus incubated with human ECs pretreated with 500 U/ml IFN-γ alone or in combination with 10 μM simvastatin. T cell proliferation could be blocked by anti-MHC class II monoclonal antibodies (data not shown). As shown in Figure 5, [^{3}H]thymidine incorporation was significantly reduced after pretreatment with simvastatin, indicating a lower T lymphocyte proliferation in response to ECs pretreated with the statin. This simvastatin-induced reduction was markedly inhibited by L-mevalonate.

Knowing that HMG-CoA reductase inhibitors upregulate endothelial nitric oxide synthase as well as nitric oxide secretion [19], we investigated the possibility that effects of simvastatin on MHC class II expression are mediated by this mechanism. Indeed, preliminary results showed that nitric oxide donors inhibited MHC class II expression and that the simvastatin-induced repression of MHC class II was partially reversed by nitric oxide scavengers (data not shown).

Discussion

Chronic rejection caused by cardiac allograft vasculopathy (CAV) is the principal cause of late death after heart transplantation [1]. The mechanisms responsible for CAV are thought to be immunological, although direct evidence is still lacking [3]. The beneficial effects of HMG-CoA re-

ductase inhibitors are usually assumed to result from their ability to reduce cholesterol synthesis [8]. However, because mevalonate is not only the precursor of cholesterol but also of many non-steroidal isoprenoid compounds, inhibition of HMG-CoA reductase may result in pleiotropic effects [14]. Hence statins, through the inhibition of HMG-CoA reductase, could affect several processes that may help to explain their non-lipid related properties. Indeed, recent *in vitro* findings indicate that these drugs may also have anti-inflammatory properties and thus may regulate important molecules in vascular biology [14]. Because some clinical studies have demonstrated a reduction in the incidence of CAV in patients under pravastatin or simvastatin treatment [12, 13], we evaluated *in vitro* the effect of simvastatin on IFN-γ induced MHC class II expression in primary human endothelial cells.

As demonstrated by flow cytometry, simvastatin dose-dependently inhibits IFN-γ induced MHC class II expression. The lower concentration of simvastatin (0.2 μM) used in this study is within the range of expected tissue levels derived from prescribed pharmacological dosages [20, 21]. MHC class I expression is almost unaffected by the statin, indicating specific effects of the drug on the MHC class II gene activation pathway. The effect of simvastatin on MHC class II expression is abolished in the presence of L-mevalonate, indicating that inhibition of HMG-CoA reductase is responsible for the repression of MHC class II. Regulation of expression of MHC class II genes is highly complex and its precise control directly influences T-lymphocyte activation and thus the immune response. Analysis of cell lines from patients suffering from a rare hereditary disease of MHC class II regulation (bare lymphocyte syndrome or MHC class II deficiency), greatly helped to understand the complex regulation of this gene [4]. Four groups of patients, all with an identical clinical picture of severe primary immunodeficiency, were shown to be affected genetically in one of four distinct transacting regulatory factors essential for MHC class II gene transcription. Whereas three of them, i.e. RFX5, RFX-AP and RFX-ANK, are ubiquitously expressed factors [4, 22], CIITA appeared to be the general controller of MHC class II expression and furthermore its own expression is tightly regulated [5, 6]. As demonstrated by RNAse protection assay, induction of MHC class II mRNA by IFN-γ is repressed by simvastatin, an effect that occurs in parallel with reduced induction of CIITA mRNA by IFN-γ. This points to inhibition of induction of the CIITA gene by simvastatin. Expression of CIITA is controlled by several alternative promoters, operating under distinct physiological conditions [7]. CIITA promoter I controls constitutive expression in dendritic cells and promoter III controls constitutive expression in B lymphocytes, while CIITA promoter IV is specifically responsible for the IFN-γ inducible expression of CIITA and thus of MHC class II. As measured by transient transfection of a promoter-reporter construct, simvastatin effectively represses the functional activity of CIITA promoter IV. We have recently reported that constitutive expression of MHC class II in dendritic cells and B lymphocytes was not affected by statins [23]. Altogether, our results point to specific actions of HMG-CoA reductase inhibitors on promoter IV of the CIITA gene. It has been shown that three transacting factors, i.e. Stat-1, USF-1 and IRF-1, are required and essential for activation of CIITA promoter IV by IFN-γ [16]. Thus, reduction of mevalonate following statin treatment may influence either the binding and/or the availability (synthesis) of these transacting factors. We have recently demonstrated that phosphorylation of Stat-1 was not affected by atorvastatin [23]. Currently, other possible mechanisms are under investigation.

MHC class II molecules play a critical role in the induction of immune responses by presenting fragments of foreign antigens to CD4+ T lymphocytes, resulting in their activation and proliferation. As measured by [^{3}H]thymidine incorporation, treatment of human ECs with simvastatin reduces T lymphocyte proliferation, thus illustrating the functional consequence of inhibition of MHC class II antigens by the drug.

In summary, we demonstrate in this study that the HMG-CoA reductase inhibitor simvastatin represses MHC class II antigen induction by IFN-γ in primary human endothelial cells and describe the mechanism of this effect via repression of promoter IV of the MHC class II transactivator CIITA. Knowing the role of endothelial immune activation in the pathogenesis of CAV, our findings may represent one of the important mechanisms by which drugs of this widely used type exert their beneficial effect after cardiac transplantation. *In vivo* studies will be needed to confirm the repression of MHC class II antigen by simvastatin. As there is increasing evidence that immuno-inflammatory interactions play important roles during atherogenesis, our results may also help to understand some of the beneficial non-lipid effects attributed to statins in the prevention of ischaemic heart diseases.

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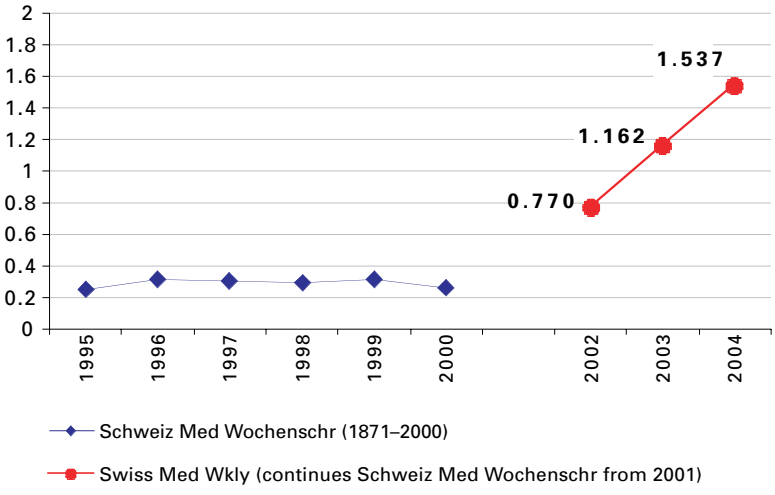

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