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Effect of statins on fibroblasts from human nasal polyps and turbinates

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Key words

Airway remodelling, fibroblasts, nasal polyp, statins, turbinate

Summary

Background: *Statins are serum cholesterol-lowering agents used for the prevention and treatment of atherosclerotic vascular disease. There is, however, growing evidence that statins have immunomodulatory and anti-inflammatory activities and may prove invaluable in the treatment of immunological and inflammatory disorders.* ***Objective:*** *On these basis we evaluated the effect of statins on the proliferation of fibroblasts derived from human nasal polyps and turbinates and determined their ability to modulate airway remodelling.* ***Methods:*** *Fluvastatin (0.01-0.1-1 μM), Atorvastatin (0.1-1-10 μM) and Simvastatin (0.1-1-10 μM) were tested on cultured fibroblasts derived from human nasal polyps and turbinates stimulated or not with Fibroblast Growth Factor β (10 ng/ml). All cultures were treated with ^{3}H-Thymidine (1 μCi/ml) to test cell proliferation.* ***Results:*** *Our results show that proliferation of turbinate-derived fibroblasts is significantly inhibited by the three statins. Fluvastatin is already effective at the lowest dose (0.01 μM), whereas Atorvastatin and Simvastatin act at the plasmatic peak concentration (1 μM). No significant effect was found on fibroblasts derived from nasal polyps, except for Simvastatin which was effective after 144 hours of stimulation. Conclusions: These drugs show a remarkable antiproliferative effect and their different outcome depending on the different kind of fibroblasts* in vitro *is prompting news in the studies about statin use for the treatment of chronic inflammatory diseases.*

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Abbreviations: IFNγ, interferon-gamma; MHC, Major Histocompatibility Complex; FBS, Fetal Bovine Serum; FGF, Fibroblast Growth Factor; DMSO, dimethyl sulfoxide; SE, standard error; COPD, Chronic Obstructive Pulmonary Disease

Introduction

Statins, the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, are effective serum cholesterol-lowering agents used in clinical practice for the prevention and the treatment of atherosclerotic cardiovascular diseases (1).

However, the observed clinical benefit with statin therapy is much greater than expected through the reduction of cholesterol levels alone (2). Clinical studies and *in vitro* experiments show, in fact, that these drugs may have beneficial effects in a broad range of inflammatory conditions.

Besides, there is growing evidence that statins have the potential to modify T lymphocyte-driven disease through the ability to inhibit the interaction among adhesion molecules (3), reduce cytokine expression, mobilize endothelial progenitor cells, interact beneficially with the renin-

angiotensin system (4-7) and decrease IFNγ-induced expression of MHC-II on Antigen Presenting Cells (8, 9).
The mechanisms responsible for these anti-inflammatory effects are currently still widely unknown.
The nasal mucosa is the first line of defense against pathogenic and non-pathogenic antigens in the air: normal breathing is through the nose and most particles are filtered there (10). The nasal mucosa has more or less the same histology as the bronchial mucosa except for the muscle layer which is present only in the bronchi. If there are any defects in these mechanical barriers, the respiratory mucosa can be injured and subsequently an inflammatory reaction will follow (11). During the latter the inflammatory cells recruited into the airways release products able to damage the surface epithelium and the underlying interstitial tissues. They also stimulate fibroblast proliferation and the extracellular matrix component deposition below the epithelial basement membrane (12).
These alterations, which include also transformation of fibroblasts into myofibroblasts, are commonly known as part of airway remodelling (13). Consequently, any treatment directed to suppress these inflammatory responses may have therapeutic benefits.
In some studies statins were able to reduce α-SMA expression and *in vitro* proliferation of fibroblasts derived from different organs, including the lower airways (14-18). On the other hand, a recent clinical report demonstrated that statins may induce nasal polyposis (19).
Considering that little is known about statin effects on upper airways, the aim of our study was to determine whether Fluvastatin, Simvastatin or Atorvastatin might inhibit proliferation of fibroblasts derived from human healthy turbinates, assumed as control fibroblasts, and nasal polyps, assumed as triggered cells.

Methods

Preparation and proliferation of nasal primary fibroblasts

Fibroblasts were obtained from nasal polyps of six patients and from turbinates of seven healthy subjects. None of the patients received anti-histamines and anti-inflammatory drug treatment (including nasal steroids) during a minimum of 2 weeks before cells were isolated. No difference in age, allergy and tobacco smoke was recorded in the two groups. None of them was on immunotherapy.
Tissues derived from nasal polyps and turbinates were cut into small fragments and incubated in RPMI-1640 (Euroclone, Milan, Italy) medium containing a mixture of 10 UI/ml DNAse, 500 UI/ml collagenase type IV and 30 UI/ml hyaluronidase (all enzymes purchased from Sigma-Aldrich, Milan, Italy) on a magnetic stirrer for 2 hours at 37°C. The cells were then cultured in RPMI supplemented with 10% FBS, glutamine and antibiotics (Euroclone, Milan, Italy) for at least 24 hours. The fibroblast cultures were characterized by flow cytometry using the specific antibody CD90 (Instrumentation Laboratory, Milan, Italy). All cultures used in the study were >95% $CD90^+$.
Fibroblasts were plated in 96-well microtitre plates at a density of approximately $5x10^3$ cells/well in 0.2 ml of RPMI plus 10% FBS and allowed to attach. After 24 hours of incubation the medium was replaced by 0.2 ml RPMI FBS-free. The day after the medium was replaced with RPMI 2% FBS and different concentrations of Fluvastatin (0.01-0.1-1 μM), Simvastatin (0.1-1-10 μM) or Atorvastatin (0.1-1-10 μM) were added. FGF (10 ng/ml) was used as positive control. Statins were tested both alone and in combination with FGF for 48, 96 and 144 hours. Cell viability was assessed by trypan blue dye exclusion and resulted >85% in all experiments. ^{3}[H]-thymidine was added during the last 18 h. The medium was then removed, the cells harvested and thymidine incorporation was measured by a beta-counter and expressed in Count per minute (Cpm). All experiments were performed in triplicate.

Statins

Fluvastatin, Simvastatin (used in the active form as sodium salt) and Atorvastatin were dissolved in DMSO to a high concentration stock solution, then further diluted in DMSO to obtain a 1000-fold higher range of concentrations than the final working one which contains 0.1% DMSO. In every set of experiments fibroblasts from three wells were treated with 0.1% DMSO alone in order to evaluate its effects on cells.

Statistical Analysis

Data are expressed as mean ± SE. Data were analyzed by non-parametric Wilcoxon test. Data were considered significant when the p value was <0.05.

Results

We investigated the efficacy of different statins (Fluvas-

tatin, Simvastatin and Atorvastatin) on fibroblast primary culture.
For this purpose we tested proliferation of turbinate and nasal polyp fibroblasts using different incubation-times (48, 96, 144 hours).
In all experiments the FGF-induced proliferation was significantly increased, compared to non-stimulated cell cultures ($p < 0.05$). Proliferation of cells stimulated with 0.1% DMSO did not statistically differ from non-stimulated cell cultures.
In preliminary experiments the most effective concentration was 0.1 μM for Fluvastatin and 1 μM for Simvastatin and Atorvastatin (data not shown) and consequently these were the concentrations of drugs used in all following set of experiments.

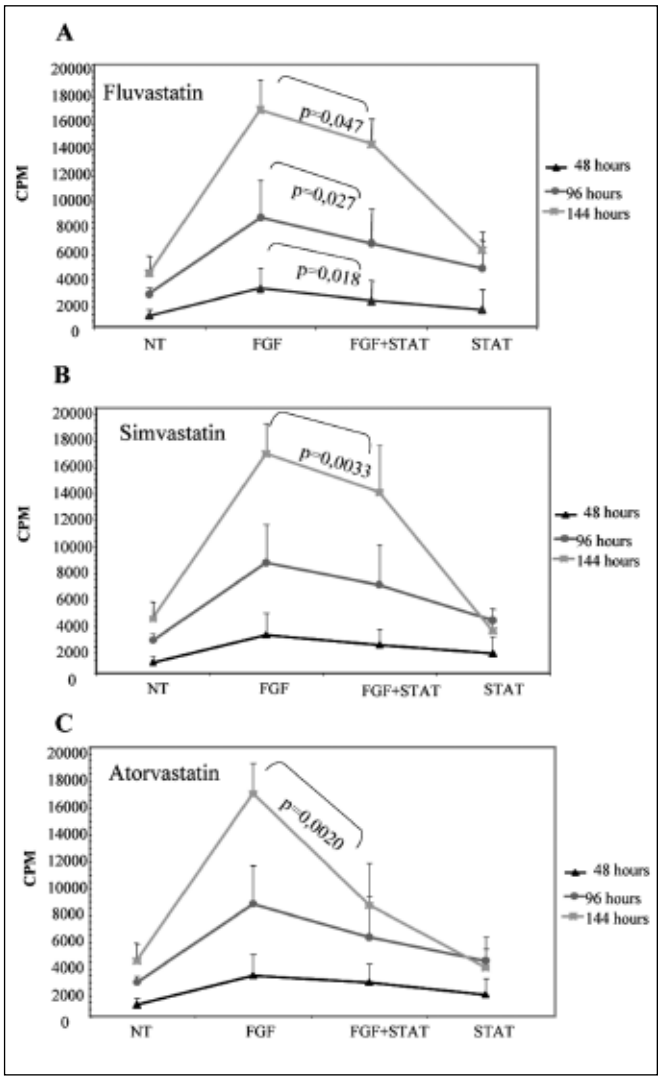
Fibroblasts from turbinates

Fluvastatin 0.1 μM significantly inhibited FGF-induced proliferation of fibroblasts derived from turbinates, when tested at 48 (p=0.018), 96 (p=0.027) and 144 hours (p=0.047).
Simvastatin and Atorvastatin 1 μM were significantly effective on turbinate fibroblast cultures carried on for 144 hours (p=0.0033 and p=0.0020 respectively) (Fig. 1).

Fibroblast from nasal polyps

Statins did not show any response on nasal polyp fibroblast proliferation. Interestingly Simvastatin 1 μM at 144 hours, however, had a significant effect (p=0.0018) (Fig. 2).
We compared the response of turbinate and nasal polyp fibroblasts to each statin to verify the effects of these drugs both in normal conditions and in chronic inflammation.
Our results demonstrated that Fluvastatin and Atorvastatin had a higher anti-proliferative effect on turbinates than on nasal polyps (Tab. 1).
It is, however, interesting to evidence the effect of Simvastatin on nasal polyps too. This drug, in fact, shows a more remarkable anti-proliferative effect on turbinates and no effect on nasal polyps, except for 144 hours incubation-time (Tab.1).

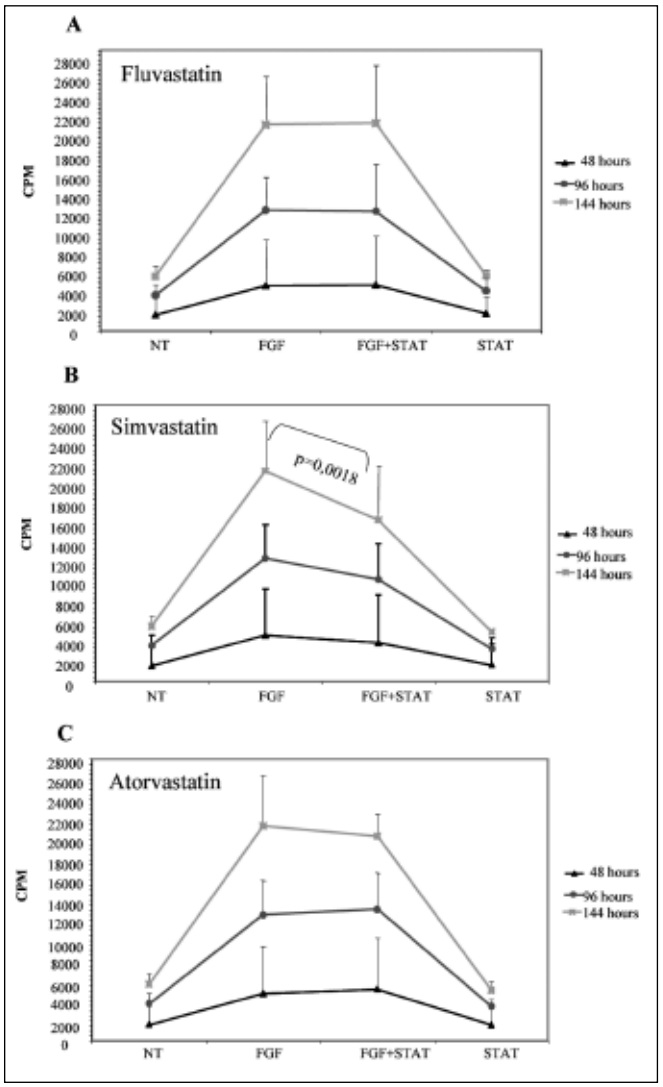
Figure 1 - Effect of Fluvastatin,Simvastatin and Atorvastatin on proliferation of turbinate fibroblasts. A) Fluvastatin; B) Simvastatin; C) Atorvastatin. Data are expressed as mean ± SD. Wilcoxon test was used for statistical analysis and data were considered significant when the p value was <0.05



Discussion

Statins are widely prescribed in cardiovascular disease patients and in view of their pleiotropic anti-inflammatory properties, not related to their cholesterol lowering activity (2), a possible use in other chronic inflammatory diseases may be envisaged. Nowadays quite a global view of some chronic disorders, such as atherosclerosis, suggests that a more general approach also for treatment should be considered. Actually, Simvastatin was shown capable of downregulating human lung fibroblast proliferation and differentiation, α-actin expression and α-actin mRNA expression, in pulmonary idiopathic fibrosis (17, 20). If such an effect is also detectable in asthma,

Figure 2 - Effect of Fluvastatin,Simvastatin and Atorvastatin on proliferation of nasal polyp fibroblasts. A) Fluvastatin; B) Simvastatin; C) Atorvastatin. Data are expressed as mean ± SD. Wilcoxon test was used for statistical analysis and data were considered significant when the *p* value was <0.05



it might be conceivable to think about if, when and how to use these drugs in asthma patients, although the very recent report by Menzies et al. (21) suggests no anti-inflammatory activity of Simvastatin in such patients. Another lung disease which might become a potential target of statins is COPD, this also considering the age of COPD patients and the frequency of cardiovascular comorbidities.

Surprisingly, a recent clinical well-documented report (19) clearly highlighted the relationship between statin assumption and nasal polyposis appearance. This could be seen as a contra-indication in using statins in subjects susceptible to develop nasal polyposis, but at present there is still no method to predict susceptibility to nasal polyposis. Because of this conflicting issue, we designed the present study to investigate statin potential effects on fibroblasts, one of the structural components of nasal polyps.

After one week of culture, an inhibitory effect of statins was consistent through all the experiments performed with turbinate fibroblasts, as far as the spontaneous and induced proliferation is concerned. However, Fluvastatin already showed an earlier inhibitory effect at 48 hours. These experimental data are of interest since a one-week culture is more similar to the long statin-cell interaction *in vivo*. Shorter culture experiments were performed to evaluate the possible fast effect of statins, which was just the case of Fluvastatin.

No effect on nasal polyp fibroblasts has been detected, except for the effect of Simvastatin after a one-week culture. Our experimental evidences suggest a class related effect of statins in controlling turbinate fibroblast proliferation, which is not the case of the same type of cells derived by nasal polyps. This might imply statins are effective in controlling proliferation of "normal" nasal fibroblasts (the ones derived from turbinates), but they are almost ineffective on nasal polyp derived fibroblasts. Moreover, their pleiotropic effects, already observed in previous studies (2, 3, 8, 22), demonstrate that the use of one type of statin may induce different results with respect to another compound of the same class.

Our report reveals the different behaviour of fibroblasts depending on their origin and is the first to investigate different statins and their effects on upper airways fibroblasts. Bearing in mind the report by Bucca and coworkers (19), the question was: are statins inducing nasal polyposis or have they no control on cell proliferation? On the basis of our study, since fibroblasts are a component of polyps, we may exclude the first possibility. In addition, we do not have evidence of an inhibitory effect on nasal polyp fibroblast proliferation, except for a marginal one exerted by Simvastatin.

We know fibroblasts are just one of the cell components of nasal polyps but in light of the published literature concerning statins and fibroblasts in lower airways, we considered it interesting to investigate these cells in our experimental model, although being aware of the fact that results obtained in animal models and *in vitro* may differ from those obtained in human beings.

The conclusions on the basis of the herein reported data

Table 1 - Resumptive scheme of Fluvastatine, Simvastatine and Atorvastatine effect on nasal polyp and turbinate fibroblasts

	FGF/FGF+Statin	Inhibition of proliferation	
Fluvastatin			
48 hours	CPM	%	↑
Nasal polyps	4633,94/4712,99	-1,7%	↓
Turbinates	2888,92/1949,16	+32,5%	
96 hours	CPM	%	
Nasal polyps	7825,83/7641,46	+2,3%	↓
Turbinates	8327,5/6342,5	+23,8%	↓
144 hours	CPM	%	
Nasal polyps	8841,66/9081,66	-2,7%	↑
Turbinates	16514/13894,16	+15,8%	↓
Simvastatin			
48 hours	CPM	%	
Nasal polyps	4633,94/4712,99	+16,0%	↓
Turbinates	2788,92/2142,92	+25,8%	↓
96 hours	CPM	%	
Nasal polyps	7825,83/7641,46	+17,4%	↓
Turbinates	8327,5/6649,55	+20,1%	↓
144 hours	CPM	%	
Nasal polyps	8841,66/6012,75	+31,9%	↓
Turbinates	16514,75/13584,58	+17,7%	↓
Atorvastatin			
48 hours	CPM	%	
Nasal polyps	4667,36/5123,09	-9,7%	↑
Turbinates	2981,78/2440,73	+18,1%	↓
96 hours	CPM	%	
Nasal polyps	7825,83/7930,96	-1,3%	↑
Turbinates	8327,5/6649,55	+29,4%	↓
144 hours	CPM	%	
Nasal polyps	8841,66/7272,16	+17,7%	↓
Turbinates	16514,75/13584,58	+50,1%	↓

CPM= Count per Minute

are: a) other mechanisms, besides fibroblast proliferation, are involved in nasal polyps induced by statins; b) statins are effective drugs in inhibiting resting or normal upper airway fibroblasts. This may not be the case of fibroblasts chronically triggered by inflammatory events, as in nasal polyposis; c) the use of statins in asthmatic patients, needing statins for a comorbidity, may not be contra-indicated but it may be even favourable if the positive effect seen in pulmonary idiophatic fibrosis is valid in asthma too. These drugs, in fact, might exert an effect on airway remodelling, namely on fibroblast to myofibroblasts differentiation. More studies should be designed to explore mechanisms of action, differences among statins and statins role in COPD.

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