Effects of simvastatin and rosuvastatin on RAS protein, matrix metalloproteinases and NF- κ B in lung cancer and in normal pulmonary tissues

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Abstract

Objectives: In this study, we have evaluated effects of 24-hour treatments with simvastatin or rosuvastatin on RAS protein, NF- κ B and MMP expression in LC tissues obtained from 12 patients undergoing thoracic surgery.

Materials and methods: Normal and lung tumour tissues obtained from each sample were exposed to simvastatin (2.5–30 μ M) or rosuvastatin (1.25–30 μ M) and western blot analysis was then performed.

Results: We documented increased expression of proteins, MMP-2, MMP-9 and NF- κ B-p65 in LC tissues, with respect to normal tissues ($P < 0.01$). In the malignant tissues, simvastatin and rosuvastatin significantly ($P < 0.01$) and dose-dependently reduced RAS protein, MMP-2/9 and NF- κ B-p65 expression.

Conclusions: In conclusion, our results suggest that simvastatin and rosuvastatin could play a role in LC treatment by modulation of RAS protein, MMP-2/9 and NF- κ B-p65.

Introduction

Lung cancer (LC) is one of the most common malignancies worldwide (1), also characterized by high mortality levels, probably attributable to early metastasis. Tobacco smoking is the most important risk factor for LC, and

accurate epidemiological studies have extensively analysed this risk, showing that it is correlated with smoking frequency (number of cigarettes smoked per day), smoking duration, and cumulative dose (pack-years) (2,3). Detection of non-invasive biomarkers can now make it possible to identify subgroups of smokers at higher risk of tobacco-induced lung damage (4). Several experimental studies have shown that age at smoking initiation is inversely associated with DNA adduct level (5) and loss of heterozygosity of chromosomes in lung tissue (6); this DNA damage is strongly related to LC risk.

Compared to normal subjects, smoker patients with LC exhibit significantly higher plasma levels of matrix metalloproteinases (MMP)-2 and -9 (7), whose expression and enzymatic activity are associated with metastatic potential of neoplastic cells (8). However, causes other than smoking can potentially be involved in development of LC. For example, Xu *et al.* (9) have reported that nickel-induced LC cell lines have increase in metastatic potential in a dose-dependent manner, accompanied by elevated expression of MMP-2 and -9, thereby suggesting a role for these MMPs in tumour progression and metastasis. Experimental studies performed with LC cells have also demonstrated that phorbol 12-myristate 13-acetate (PMA) may affect expression of MMPs by regulation of transcription factors such as nuclear factor- κ B and activator protein-1 (AP-1), through PI3K/Akt, JNK, p38 MAPK, and ERK signalling pathways (10,11).

A number of chemotherapeutic drugs is used in LC management, albeit that the clinical outcome of patients is poor. Recent studies have shown that antioxidant compounds may be safe and effective anti-cancer agents for LC therapy (12), in particular, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins) exert pleiotropic effects (for example, anti-inflammatory and antioxidant properties), independently

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from their ability to reduce circulating low-density lipoprotein cholesterol (13).

Currently, there is growing interest in statins due to their possible anti-cancer effects (14), indeed they have anti-proliferative, proapoptotic, anti-invasive and radiosensitizing properties (15). They inhibit the mevalonate pathway, thus leading to critical changes in several cell functions, especially related to post-translational modifications of the oncogenic protein. In particular, RAS activation requires covalent attachment of a non-sterol isoprenoid consisting of either farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP) (16). By inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme within the mevalonate pathway, statins also block synthesis of downstream mevalonate derivatives FPP and GGPP (17). In this regard, it is noteworthy that simvastatin can inhibit population growth of small cell lung cancer (SCLC) cells, and this effect is likely to be due to interference with RAS-dependent activation of the MEK/ERK signalling complex (18). Moreover, statins may have a cytostatic effect on cancer cells, thus be useful to prolong survival of cancer patients (19), and also act as antioxidant, anti-inflammatory and anti-angiogenic factors, all being able thus, to prevent or inhibit malignant cell growth (20). Furthermore, we have recently shown that simvastatin is able to induce reduction in cell proliferation and significant increase in apoptosis of non-small cell lung cancer (NSCLC) cell cultures. In contrast, Kuoppala *et al.* (22) have suggested that statins do not have any short-term effect on

cancer, but we find no strength in their evidence. In particular, their conclusions are mostly based on small observational reports, often not corroborated by larger or randomized studies (23).

On the basis of the above-mentioned considerations, in this study, we have evaluated effects of 24-hour treatments, with simvastatin or rosuvastatin on RAS protein, MMP and NF- κ B expression, in LC tissues obtained from patients undergoing therapeutic surgery.

Materials and methods

Study design

Patients with LC scheduled for surgery, were recruited into an open-label, single-centre study at “Pugliese-Ciacio” Hospital of Catanzaro, Italy, between January 2010 and June 2012. The study was approved by the Research Ethics Committee (EUDRACT number 2010-019530-27), and was carried out in accordance with the Declaration of Helsinki and Guidelines for Good Clinical Practice. All participants signed written informed consent documents prior to enrolment.

Tissue isolation

Lung tissues, obtained from fresh surgical specimens taken from each patient undergoing either lobectomy or pneumonectomy for LC (Fig. 1), were placed in saline solution. Portions of the surgical samples were used for histopathological evaluation.

Figure 1. Experimental protocol for both malignant and non-malignant tissue isolation from patients with LC.

Normal and neoplastic lung tissues as defined by visual inspection, were weighed, and totals of 0.4 g each were cultured in Minimum Essential Medium containing 10% foetal bovine serum (FBS), with added antibiotics (100 U/ml of penicillin and 100 mg/ml of streptomycin; Sigma Aldrich, Milan, Italy) and Fungizone (1 mg/ml; Gibco-BRL, Gaithersburg, MD, USA). After 12 h, tissues were exposed to 24-hour treatment, with increasing doses of simvastatin (2.5–30 μ M) or rosuvastatin (1.25–30 μ M).

After treatment, tissues were lysed for western blot analysis in 2 ml of tissue protein extraction reagent (25 mM Bicine, 150 mM sodium chloride pH7.6; Thermo Scientific, Rockford, IL, USA). Protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) and lysates were stored at -80°C . Only tissues for which detailed histopathological examination confirmed initial visual diagnosis of non-small cell LC and, as control, neighbouring healthy tissues obtained from the same patient, were subjected to further study (Fig. 1).

For western blotting, whole cell and nuclear extracts were separated on 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes as previously described (24–26). Immunoblotting was performed using anti-RAS protein, MMP-2, MMP-9 and NF- κ B-p65, monoclonal antibodies.

Antibody binding was visualized using enhanced chemiluminescence; intensities of experimental bands were analysed by computer-assisted densitometry as pre-

viously described (27), and expressed as arbitrary units (AU).

All experiments were performed in triplicate.

Electrophoretic mobility shift assay (EMSA)

NF- κ B activation was monitored by binding to the high affinity HIV-1 NF- κ B binding site formed by annealing two oligonucleotides 5'-CAAGGGACTTTCCGCTGGG GACTTTCCAG-3' and 5'-CTGGAAAGTCCCCAGC GGAAAGTCCCTTG-3'. Briefly, the probe was end-labelled with [γ - ^{32}P]ATP 3000 Ci/mmol (Amersham

Table 1. Characteristics of recruited patients. Cancer grading performed through histological evaluation

Patient	Sex	Age	Smoke exposure	Cancer histology	Cancer grading
1	M	54	Smoker	Adenocarcinoma	G4
2	M	61	Ex-smoker	Adenocarcinoma	G3
3	F	66	Smoker	Adenocarcinoma	G2
4	M	65	Smoker	Adenocarcinoma	G3
5	F	57	Smoker	Adenocarcinoma	G3
6	F	70	Ex-smoker	Adenocarcinoma	G4
7	M	72	Smoker	Adenocarcinoma	G2
8	M	67	Ex-smoker	Adenocarcinoma	G3
9	M	67	Smoker	Adenocarcinoma	G2
10	F	62	Smoker	Adenocarcinoma	G2
11	M	75	Ex-smoker	Adenocarcinoma	G2
12	F	58	Smoker	Adenocarcinoma	G2

G2: intermediate differentiation; G3: low differentiation; G4: poor differentiation.

Figure 2. Representative immunohistochemical staining of TTF-1 (thyroid transcription factor-1) (a), cytochrome-7 (b), cytochrome-8 (c) and cytochrome-20 (d) in LC tissues of enrolled patients.

Int., Buckinghamshire, UK) using polynucleotide kinase (Promega, Madison, WI, USA). Equal amounts (5 µg) of nuclear extract were incubated in 20 µl reaction mixture containing 10% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 2 µg of poly [d(G-C)] (Promega) for 5 min on ice. One microlitre of [γ - 32 P]-labelled double-stranded probe (0.2 ng, 5×10^4 cpm) was then added, with or without 100-fold molar excess of competitor oligonucleotide. Reactions were incubated at room temperature for 20 min and run on a 6% acrylamide:bisacrylamide (30:1) gel in 22.5 mM Tris borate, 0.5 mM EDTA. Gels were dried and autoradiographed.

Figure 3. RAS expression in healthy and LC tissues, in presence or absence of a 24-h treatment with simvastatin (30 µM). Data referring to one patient are shown. ** $P < 0.01$.

Immunohistochemical assay of LC molecular markers

Four micrometre serial sections from the formalin-fixed, paraffin-embedded lung tissue blocks were immunostained for thyroid transcription factor-1 (TTF-1), cytokeratine-7, cytokeratine-8 and cytokeratin-20, using avidin-biotin complex immunoperoxidase methods. Sections were examined using light microscopy, by two independent investigators, blind to content of the treatment groups. Representative areas of each section were selected, and cells were counted under high magnification [400 $\times$] 10 fields total. For each section, immunoreactivity was quantified based on percentage of positive specific cells over total specific cells examined. Cells were considered positive if they stained in brown reaction product above background staining.

Statistical analysis

In this study, our results are exploratory, thus, we have not performed power calculations, but all data are expressed as mean $\pm$ standard deviation (SD). Statistical evaluation of results was performed by analysis of variance (ANOVA), differences identified and pinpointed by unpaired Student's *t*-test. Threshold of statistical significance was set at * $P < 0.05$.

Results

Patients

From January, 2010 to June 2012, 12 patients - 5 women (mean age 62.60 years, SD = 5.46) and 7 men

Figure 4. RAS expression in LC tissues, in presence or absence (control) of a 24-h treatment with simvastatin at different drug concentrations (2.5–30 µM). (a) A representative western blot of one of the 12 patients enrolled in the study is shown. (b) Data of the 12 patients enrolled in the study are shown. Densitometric evaluation of western blots was performed and output was quantified as arbitrary units. Data are expressed as mean $\pm$ standard deviation. * $P < 0.05$; ** $P < 0.01$.

Figure 5. RAS expression in LC tissues, in presence or absence (control) of 24-h treatment with rosuvastatin at different drug concentrations (1.25–30 μM). (a) A representative western blot of one of the 12 patients enrolled in the study is shown. (b) Data of the 12 patients enrolled in the study are shown. Densitometric evaluation of western blots was performed, and output was quantified as arbitrary units. Data are expressed as mean ± standard deviation. * $P < 0.05$; ** $P < 0.01$.

Figure 6. MMP-2 expression in healthy and LC tissues, in presence or absence of 24-h treatment with simvastatin (30 μM). Data referring to one patient are shown. ** $P < 0.01$.

Figure 7. MMP-9 expression in healthy and LC tissues, in presence or absence of 24-h treatment with simvastatin (30 μM). Data referring to one patient are shown. ** $P < 0.01$.

(mean age 65.86 years, SD = 6.94), were enrolled in the study and completed the informed consent process. All enrolled patients had been smokers for over 10 years (Table 1).

Evaluation of LC molecular markers

In malignant tissues, positive, intense brown staining for TTF-1, cytokeratine-7, and cytokeratine-8 was detected, whereas no staining was observed for cytokeratine-20. Taken together, these data indicate presence of primary adenocarcinoma of the lung (Fig. 2).

RAS protein expression

Lung cancer tissues had higher levels of RAS protein expression compared to normal tissues (LC tissues: 153 ± 0.1 AU; normal tissues: 55 ± 0.08 AU). Treatment with simvastatin (30 μM) significantly reduced RAS expression in LC tissues (60 ± 0.08 AU), but not in normal ones (57 ± 0.05 AU) ($P < 0.01$) (Fig. 3). Moreover, 24-h treatments with either simvastatin or rosuvastatin induced significant and dose-dependent reduction ($P < 0.01$) in RAS expression (Figs 4,5).

MMP-2 and MMP-9 expression

Our results indicated increased expression of both MMP-2 and MMP-9 in LC tissues, compared to normal tissues (MMP-2: cancer tissue: 153 ± 0.15 AU; normal tissue 52 ± 0.09 AU; MMP-9: cancer tissue: 100 ± 0.12 AU; normal tissue: 18 ± 0.05 AU) ($P < 0.01$) (Figs 6,7). Both MMP-2 and MMP-9 levels were reduced by 24-hour treatment with simvastatin ($30 \mu\text{M}$) in cancer tissues (MMP-2: 46 ± 0.03 AU; MMP-9: 48 ± 0.04 AU) ($P < 0.01$), but not in normal ones (MMP-2: 14 ± 0.07 AU; MMP-9: 15 ± 0.05 AU) (Figs 6,7).

In malignant tissues, 24-h treatments with either simvastatin or rosuvastatin induced significant and dose-dependent reduction ($P < 0.01$) for both MMP-2 (Figs 8,9) and MMP-9 expression (Figs 10,11).

NF- κ B-p65 expression

Western blot analysis of nuclear extracts demonstrated higher expression levels of NF- κ B-p65 in LC tissues, compared to normal tissues (100 ± 0.06 AU and 63 ± 0.03 AU, respectively) (Fig. 12). Treatment with

Figure 8. MMP-2 expression in LC tissues, in presence or absence (control) of 24-h treatment with simvastatin at different drug concentrations (2.5–30 μM). (a) Representative western blot of one of the 12 patients enrolled in the study is shown. (b) Data of the 12 patients enrolled in the study are shown. Densitometric evaluation of western blots was performed and output quantified as arbitrary units. Data are expressed as mean $\pm$ standard deviation. * $P < 0.05$; ** $P < 0.01$.

Figure 9. MMP-2 expression LC tissues, in presence or absence (control) of 24-h treatment with rosuvastatin at different drug concentrations (1.25–30 μM). (a) Representative western blot of one of the 12 patients enrolled in the study is shown. (b) Data of the 12 patients enrolled in the study are shown. Densitometric evaluation of western blots was performed and output was quantified as arbitrary units. Data are expressed as mean $\pm$ standard deviation. * $P < 0.05$; ** $P < 0.01$.

Figure 10. MMP-9 expression in LC tissues, in presence or absence (control) of 24-h treatment with simvastatin at different drug concentrations (2.5–30 μM). (a) Representative western blot of one of the 12 patients enrolled in the study is shown. (b) Data of the 12 patients enrolled in the study are shown. Densitometric evaluation of western blots was performed and output was quantified as arbitrary units. Data are expressed as mean ± standard deviation. * $P < 0.05$; ** $P < 0.01$.

Figure 11. MMP-9 expression in LC tissues, in presence or absence (control) of a 24-h treatment with rosuvastatin at different drug concentrations (1.25–30 μM). (a) Representative western blot of one of the 12 patients enrolled in the study is shown. (b) Data of the 12 patients enrolled in the study are shown. Densitometric evaluation of western blots was performed and output was quantified as arbitrary units. Data are expressed as mean ± standard deviation. * $P < 0.05$; ** $P < 0.01$.

simvastatin (30 μM) for 24 h significantly ($P < 0.01$) reduced NF-κB-p65 expression in both LC and normal tissues (55 ± 0.07 AU and 26 ± 0.02 AU, respectively) (Fig. 12).

In LC tissues, both simvastatin and rosuvastatin induced significant and dose-dependent reduction in NF-κB-p65 expression ($P < 0.01$) (Figs 13,14). This correlated with significant reduction in DNA binding activity (Fig. 15), as detected by electrophoretic mobility shift assays (EMSAs) as described in the Materials and methods section.

Discussion

In this study, we have evaluated effects of simvastatin and rosuvastatin on LC and normal pulmonary tissues, with regard to RAS protein, MMP-2, MMP-9 and NF-κB-p65 expression. MMPs are known to promote cancer progression by extracellular matrix and basement membrane degradation, resulting in exposure of cryptic anatomical locations linked to invasion, metastasis of malignant cells and to angiogenesis (28–30).

In particular, MMP-2 and MMP-9 expressed by cells of various malignant tumours are closely related to invasive and metastatic properties of the cells, and also play a critical role in degradation of type-IV collagen (31). The 5' flanking regions of *MMP-2* and *MMP-9* genes contain several functional regulatory motifs that bind well-characterized transcription factors, such as NF- κ B, activator protein-1, stimulatory protein-1, and polyoma-virus enhancer activator-3 (32,33). Through interactions between these transcription factors and their respective DNA binding sites, various agents including growth factors and cytokines are able to regulate expression of MMP-9 (34,35). On the other hand, NF- κ B is also

involved in a number of biological processes, including cell proliferation, differentiation, tumour progression and metastasis (36).

In the present study, western blot analysis revealed significantly higher levels of MMP-2, MMP-9 and NF- κ B in LC tissues, taken from patients with pulmonary adenocarcinoma, compared to normal tissues, derived from the same patients. We also observed that 24-hour treatment with simvastatin or rosuvastatin significantly reduced expression of NF- κ B-p65, MMP-2 and MMP-9 in malignant tissues. Furthermore, in normal lung tissues, simvastatin reduced NF- κ B-p65 levels, but not MMP-9 expression, suggesting that MMPs may be modulated only when they are over-expressed. These data confirm involvement of the HMG-CoA reductase pathway in processes of metastatic invasion related to NF- κ B and MMP activity. Cancer cells usually express high levels of HMG-CoA reductase, which appear to be required by NSCLC cells to satisfy their increased need for isoprenoids and lipids.

Capability of statins to inhibit MMP-2 and MMP-9 expression has also been demonstrated in cell lines, such as those of macrophages and smooth muscle cells (37). In these, inhibitory effects of cerivastatin on MMP-2 and MMP-9 expression was reversed by mevalonate and GGPP (37). Also, simvastatin can inhibit RAS-dependent induction of MMP-9 expression in rat alveolar macrophages exposed to cigarette smoke extracts, and this effect can be abrogated by supplementation of farnesyl pyrophosphate or GGPP (38). Thus, by blocking RAS prenylation, statins seem to interfere with NF- κ B-dependent signalling networks responsible for MMP

Figure 12. NF- κ B-p65 expression in healthy and LC tissues, in presence or absence of a 24-h treatment with simvastatin (30 μ M). Data referring to one patient are shown. ** $P < 0.01$.

Figure 13. NF- κ B-p65 expression in LC tissues, in presence or absence (control) of a 24-h treatment with simvastatin at different drug concentrations (2.5–30 μ M). (a) Representative western blot of one of the 12 patients enrolled in the study is shown. (b) Data of the 12 patients enrolled in the study are shown. Densitometric evaluation of western blots was performed and output was quantified as arbitrary units. Data are expressed as mean $\pm$ standard deviation. * $P < 0.05$; ** $P < 0.01$.

Figure 14. NF-κB-p65 expression in LC tissues, in presence or absence (control) of 24-h treatment with rosuvastatin at different drug concentrations (1.25–30 μM). (a) Representative western blot of one of the 12 patients enrolled in the study is shown. (b) Data of the 12 patients enrolled in the study are shown. Densitometric evaluation of western blots was performed and output was quantified as arbitrary units. Data are expressed as mean ± standard deviation. * $P < 0.05$; ** $P < 0.01$.

expression. Furthermore, our results show that simvastatin and rosuvastatin are also able to reduce RAS expression. Statins thus, may effectively inhibit RAS/NF-κB/MMP-mediated pathways involved in degradation of extracellular matrix and subsequent cell invasiveness, which are key steps in the metastatic process. In addition to directly stimulating *MMP-9* gene transcription, NF-κB is able to induce *MMP-9* expression by indirect mechanisms. For example, in murine keratinocyte cell lines, NF-κB has been shown to be able to stimulate production of transforming growth factor (TGF)-β1, which in turn increased *MMP-9* expression (39). It can thus be argued that through these interactive mechanisms, NF-κB and *MMP-9* play a key role in TGF-β1-induced enhancement of basement membrane degradation, metastatic invasion and overall cell malignancy. Thus, statins can potentially contribute to attenuation of invasive and metastatic properties of malignant cells. Recently, Brown *et al.* (40) documented that lipophilic statins (atorvastatin, simvastatin and rosuvastatin) reduced migration and colony formation of PC-3 prostate cancer cells in human bone marrow stroma, by inhibiting GGPP production, thus decreasing generation, and spreading of metastatic prostate colonies. In agreement with these findings, Polo *et al.* (41) reported in human hepatocarcinoma cell lines that pharmacological treatment with combination of simvastatin and geraniol resulted in significant inhibition of cell proliferation. Moreover, we have recently shown in two lines of NSCLC cells (CALU-1 and GLC-82 lines) that simvastatin was able to significantly reduce cell proliferation, and increase apoptosis, by inhibition of the MAPK-ERK pathway (21).

Figure 15. Dose-response curve of NF-κB binding activity inhibition. LC tissues from one representative patient enrolled in the study were treated with rosuvastatin and with simvastatin, as indicated below the lanes. Untreated LC tissue and healthy tissue from the same patient were used as controls. After treatment, nuclear extracts were prepared and subjected to EMSA as described in the Materials and methods section.

However, previous data have shown that statins are characterized by only modest anti-cancer properties when used as monotherapies (42). Indeed, in cancer treatment, statins are mainly considered to be potentially useful drugs when combined with conventional chemotherapeutic agents (43). With regard to LC, as 40–80% of NSCLC subtypes over-express epidermal growth factor receptor (EGFR), whose activation triggers downstream signalling networks involving the RAS-Raf-MAPK pathway, on-going experimental approaches are evaluating therapeutic potential of pharmacological associations including statins and EGFR tyrosine kinase inhibitors such as gefitinib (44,45). In particular, some *in vitro* studies have shown that

combination of gefitinib and lovastatin produces synergistic cytotoxicity in gefitinib-resistant NSCLC cells (46). A recent randomized phase II trial has demonstrated that when compared to gefitinib alone, in patients with advanced NSCLC, drug association gefitinib-simvastatin is capable of improving both tumour response rate and patient progression-free survival (47).

In conclusion, our findings referring to potential anti-tumour activities of simvastatin and rosuvastatin, detected in LC tissues with regard to inhibition of MMP-2, MMP-9 and NF- κ B expression, further corroborate usefulness of *in vivo* studies aimed to evaluate effects on LC of statins, eventually used in association with other anti-cancer drugs. Some limitations of our current study include low number of patients recruited and absence, among the enrolled subjects, of non-smokers. Despite low number of enrolled patients, our preliminary results appear to be interesting as they shed light on putative mechanisms of statins as anti-LC agents, specially concerning possible inhibition of metastatic potential of LC cells. On the basis of our findings, a logical development of the present study would be comparative evaluation, between smokers and non-smokers, of expression patterns of NF- κ B and MMP2/9 in LC tissues, with the aim of detecting differences in invasive and metastatic behaviours of LC in these two subject categories. This issue could be addressed in future investigations.

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