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Atorvastatin-Induced Inhibition of Human Melanoma *In Vivo* Development

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Abstract

Statins, 3-hydroxy 3-methylglutaryl CoA (HMG-CoA) reductase inhibitors, are pleiotropic pharmacological inhibitors, which block the mevalonate synthesis pathway. They are widely used as hypocholesterolemic agents and have shown protective effects against cancers.

Our previous data showed that statin treatment induced MHC class I chain-related protein A (MICA) membrane overexpression in human melanoma cells, which increased their sensitivity to NK cell cytotoxicity and thus the inhibition of tumor development. MICA is a ligand of the NK cell activating receptor NKG2D. This receptor is essential for NK cell control of tumor development and it has the unique property of being able to recognize tumor cells of both murine and human origin.

Here, using atorvastatin and the WM-266-4 melanoma cell line, we first confirmed that statin treatment enhances MICA membrane expression and decreases local tumor growth and pulmonary metastasis implantation *in vivo*. Furthermore our new experiments showed that atorvastatin intraperitoneal repeated injections induced a reduction of tumor growth following subcutaneous implantation of untreated WM 266-4 cells into NMRI nude mice and favored the innate anti-melanoma immune response by increasing splenic NK cell concentration and activation. This report confirms that statins could become effective pharmacological agents for melanoma immunotherapy.

Keywords: Statins; Melanoma; Metastasis; NKG2D; MHC class I chain-related protein A (MICA)

Introduction

Despite tumor antigen expression in human melanoma cells and the mobilization of an immune response in patients, survival after metastasis detection is usually short [1]. During the oncogenic process, tumor cells must overcome inherent but also micro environmental control mechanisms, among which the innate and adaptive immune responses play a major role [2]. Interestingly, some pharmacological drugs such as statins render melanoma cells more immunogenic for the both the innate and adaptive anti-tumor immune responses [3-5]. We have previously shown that the *in vitro* treatment of human melanoma cells with atorvastatin or lovastatin enhanced tumor membrane expression of the MHC class I chain-related protein A (MICA), which is a ligand of the NKG2D activating receptor of NK cells [6-8]. This effect is important because NKG2D expression is essential for NK cell control of tumor development [9]. Interestingly NKG2D can recognize tumor cells of both murine and human origin [10]. This unique property allowed us to show that the statin-induced MICA membrane overexpression not only enhanced *in vitro* melanoma sensitivity to NK cell lysis but also decreased local tumor growth and pulmonary metastasis implantation in mice injected with statin-treated melanoma cells [6].

Statins are 3-hydroxy 3-methylglutaryl CoA (HMG-CoA) reductase inhibitors. They are pleiotropic pharmacological inhibitors, which block the mevalonate synthesis pathway and consequently Ras and Rho GTPase activity [11]. They also enhance Peroxisome Proliferator-Activated Receptor gamma (PPARγ) activity [12]. Statins are used by millions of people as hypocholesterolemic agents in cardiovascular and cerebrovascular diseases and retrospective studies have shown that they could also have protective effects on the development of cancers including melanomas [13-15]. In particular, *in vitro* atorvastatin treatment has been shown to revert the metastatic phenotype of human melanoma cells, including WM266-4 cells [16]. The inhibition of Rho GTPase activity is involved in the protective effects of statins. Indeed, RhoC overexpression has been shown to dramatically increase the *in vivo* melanoma metastatic potential, and atorvastatin treatment was shown to inhibit the colonization and formation of lung metastases of melanoma cells overexpressing RhoC [17]. Unexpectedly, our study describing statin-induced MICA overexpression showed that PPARγ is involved in this regulation and is independent of the Ras and Rho GTPase signaling pathways [6].

The statin-induced inhibition of melanoma development that we described previously using pretreated LB1319-MEL and BB74-MEL

melanoma cell lines suggested that statins could become new treatment options for metastatic melanoma treatment. Here, similar results were obtained with another human melanoma cell line, WM-266-4. This cell line has allowed us to demonstrate statins ability to slow the growth of tumors derived from melanoma cells that have not been pretreated. These data are essential since in order to be considered as really useful cancer drugs statins need to be efficient *in vivo* on untreated tumor cells. In the present study we analyzed the effect of repeated intraperitoneal atorvastatin injections on reducing the growth of untreated melanoma cells. Results show that subcutaneous tumors derived from untreated WM-266-4 melanoma cells in NMRI nu/nu mice were significantly reduced by atorvastatin injections. Furthermore, atorvastatin injections enhanced splenic activated NK cells. Therefore this report provides further evidence that statins could become interesting pharmacological agents for melanoma immunotherapy.

Material and Methods

Tumor cell line and mice

WM-266-4 human melanoma cell line was obtained from the American Type Culture Collection and maintained in culture by serial passages in culture medium composed of RPMI 1640 medium (Lonza) supplemented with 10% fetal bovine serum (FCS), 1mM glutamine, 1% penicillin-streptomycin-amphotericin B (Lonza) and it was monthly tested to be mycoplasm-free.

Six- to eight-week-old female NMRI nu/nu mice were used for all experiments described in this study. They were obtained from Elevages Janvier. All experiments involving mice were done using appropriate conditions of husbandry, experimentation and care, supervised by the Ethic Comity of the Institut Claudius Regaud under the control of the Regional Comity of Midi-Pyrénées (France). Our protocol was validated and received the agreement numbers ICR-2009-0011 and ICR-2009-0020.

In vitro treatment of melanoma cells

WM-266-4 cells were either untreated or treated for 48h with one 1 or 5 µM of the synthetic statin: Atorvastatin (Pfizer).

Flow cytometric analyses

To detect membrane MICA expression 1×10^6 WM-266-4 cells were stained with PE-conjugated anti-MICA mAb and isotype control purchased from R&D Systems. 1×10^6 splenocytes were stained to detected NK cells and activated NK cells with APC-conjugated NK1.1 mAb and PE-conjugated CD69 obtained from Beckton Dickinson Biosciences. Stained cells were analyzed on a BD FACS Calibur (BD) and results were analyzed with FlowJo software (Tree Star). To evaluate membrane antigen expression on several independent experiments, the index of specific fluorescence (ISF) was determined. This was calculated using the following formula: (median fluorescence intensity (MFI) with the specific antibody - MFI with the isotype control) / MFI with the isotype control × 100.

In vitro proliferation of untreated versus Atorvastatin pre-treated tumor cells

1×10^5 WM-266-4 cells either untreated or pretreated for 48 h with 1 or 5 µM of Atorvastatin were cultivated *in vitro*. WM-266-4 cells were counted after 2, 4, 6, 9 and 12 days of culture with Cell Counter (Coulter) to evaluate their *in vitro* proliferation, which allows evaluating the toxicity of Atorvastatin treatment.

Subcutaneous tumor growth

Two kinds of experiments were done:

- The first one was performed to evaluate the impact of in vitro Atorvastatin pre-treatment on local (subcutaneous) WM-266-4 melanoma tumor development. For this purpose nude mice were injected subcutaneously (s.c.) with 5 × 105 WM-266-4 cells either untreated or pre-treated with 1 or 5 µM Atorvastatin for 48 h. These cells were washed thrice in PBS before the injections to completely eliminate the statin.
- The second one was performed to evaluate the impact of repeated intra-peritoneal (IP) Atorvastatin injections on local (subcutaneous) WM-266-4 melanoma tumor development. For this purpose nude mice were injected s.c. with 5 × 105 untreated WM266-4 cells and every following day mice were IP injected with 0.1 ml of PBS or 30 µg of Atorvastatin. This dose was chosen because it corresponds to the dose given to humans as hypocholesterolemic agents in cardiovascular and cerebrovascular diseases.

In both experiments mice were monitored for tumor growth every 2-3 days by palpation and tumor surfaces were measured using digital caliper. Tumor-bearing mice were sacrificed at day 18 or 20 after tumor injection. At this time all mice were alive and the tumors did not display ulcerations. Two groups of 5 or 6 mice were tested and the experiments were done twice. Results are expressed as surface ± SEM (error bars, n=11 mice). Statistical analysis was performed using a two-way ANOVA test.

Pulmonary metastases implantation and detection

NMRI nude mice were injected intravenously (i.v.) in the tail vein with 5 × 105 WM-266-4 cells either untreated or pretreated 48 h with 1 or 5 µM Atorvastatin. Melanoma cells were washed thrice in PBS before the injections to completely eliminate the statin. Mice were sacrificed 20 days later; at this time all mice were alive. The lungs were fixed in formalin and paraffin embedded to visualize microscopic metastases. Lung metastases were quantified after immunohistochemical (IHC) staining by a pathologist and a biologist. These analyses were done independently and blind. The IHC staining was done with KBA.62 mAbs [18] ready to use from DAKO revealed by FLEX/HRP (20 min incubation) then by FLEX DAB+ Sub-Chromo (10 min incubation) and FLEX Hematoxylin (5 min incubation). Photos were taken with a DMR microscope (Leica Microsystems) and a DS-Fi1 camera (Nikon Instruments). The experiments included two mice/group and were repeated twice.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software. Significance of flow cytometry analyses was assessed by t-test or Tukey one-way ANOVA test. All statistic tests were two-sides. The values are expressed as mean values ± standard deviation (SD) or standard error of the mean (SEM) in the figures. P-values less than 0.05 were considered statistically significant.

Result

Atorvastatin treatment of WM266-4 melanoma cells induces MICA overexpression *in vitro*

Atorvastatin was chosen because of its favorable safety profiles relative to other drugs in its class [19] and because we found that mice tolerated atorvastatin treatment without any obvious toxicity. Our previous study concerning the role of statins in establishing an innate anti-melanoma immune response used two human melanoma cell lines, LB1319-MEL and BB74-MEL [6]. Here, the WM-266-4 melanoma cell line was chosen to continue this study because when these cells are injected intravenously into nude mice they systematically induce lung metastases that are easy to quantify. We first tested whether the *in vitro* treatment of these cells with a statin could, as previously observed with LB1319-MEL and BB74-MEL cells, induce an overexpression of the NKG2D ligand MICA that leads to an increased sensitivity to NK cell lysis. WM-266-4 cells were treated for 48 h with 1 or 5 µM of atorvastatin, which induced a weak but significant and reproducible increase in MICA membrane expression (Figures 1A and 1B). In these cells, lower doses of atorvastatin, such as 0.5 and 1 µM, were sufficient to induce a significant increase in MICA expression (data not shown). These data confirm that statin treatment of human WM-266-4 melanoma cells enhances MICA membrane expression.

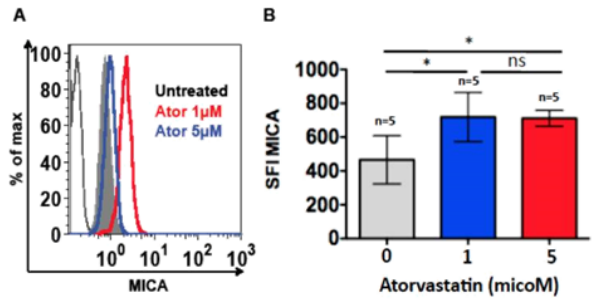

Figure 1: Atorvastatin treatment induces MICA overexpression on WM-266-4 cells. WM-266-4 cells were treated with Atorvastatin (Ator) for 48 h at indicated concentrations or not. MICA membrane expression was analyzed by flow cytometry. A representative experiment is illustrated in (A). To pool five independent experiments, the index of specific fluorescence (ISF) was calculated as indicated in the Section "Materials and Methods" (B). Results are expressed as mean ± SD [error bars, n=3 experiments (B). $^{*}P<0.05$; $^{**}P<0.005$ versus control condition using the ANOVA Tukey test.

Pre-treatment of WM-266-4 cells with atorvastatin does not slow down their proliferation *in vitro*

We next sought to confirm that atorvastatin is not toxic for WM-266-4 cells. WM-266-4 cells were untreated or pre-treated for 48 h with two doses (1 and 5 µM) of atorvastatin. Cells were then cultivated *in vitro* and counted every two days. Results show that all cells grew at similar rates, demonstrating that atorvastatin was not toxic (Figure 2).

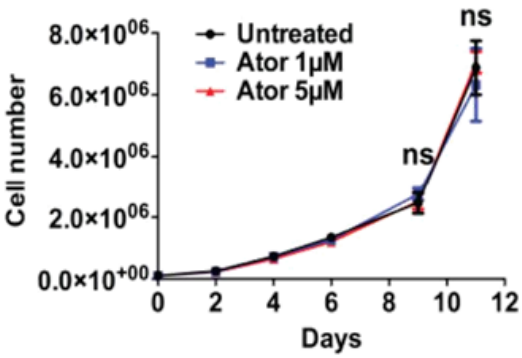

Figure 2: Atorvastatin treatment does not affect WM-266-4 cells proliferation *in vitro*. WM-266-4 cells were treated with 1 µM or 5 µM of Atorvastatin (Ator) for 48 h or untreated. The cells were then cultivated and every 2–3 days pre-treated (Ator) and untreated WM-266-4 cells were counted to evaluate the *in vitro* proliferation. Results are illustrated as mean ± SEM (error bars, n=3 experiments). ns versus control condition using the two-way ANOVA test.

Atorvastatin pre-treatment reduces local WM-266-4 melanoma growth *in vivo*

To investigate the effects of atorvastatin treatment *in vivo*, we performed subcutaneous injection of 5×10^5 WM-266-4 cells, either untreated or pre-treated for 48 h with 1 or 5 µM atorvastatin, into the flank of NMRI nude mice. The resulting tumors showed that WM-266-4 cells pre-treated with atorvastatin grew slower than untreated cells (Figure 3). These results are similar to those obtained previously with LB1319-MEL cells [6] and confirm the capacity of the statin to reduce tumor growth at nontoxic doses that induce MICA overexpression.

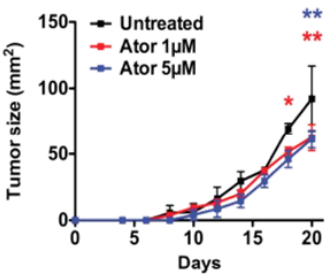

Figure 3: Atorvastatin pre-treatment reduces local *in vivo* WM-266-4 melanoma growth. 5×10^5 WM-266-4 cells untreated or pre-treated with Atorvastatin at 1 and 5 µM for 48 h (Ator) were injected subcutaneously into the flank of four NMRI nu/nu mice. Tumor growth was measured every 2-3 days. Results are illustrated as mean ± SEM (error bars, n=1 experiment). $^{*}P<0.05$; $^{**}P<0.005$ versus control condition using the two-way ANOVA test.

Atorvastatin pre-treatment reduces lung metastasis development

NMRI nu/nu mice have functional NK cells that are able to kill human MICA-positive tumor cells. Therefore, because NK cells play an important role in the control of melanoma metastatic processes [20,21], we tested whether the pretreatment of WM-266-4 cells with atorvastatin could reduce metastasis development. Intravenous injection of melanoma cells followed by lung examination allows a quantitative evaluation of the final steps in metastasis formation. Therefore, 5×10^5 WM-266-4 cells either untreated or pre-treated for 48 h with 1 or 5 µM atorvastatin were injected intravenously into the tail vein of nude mice and animals were then sacrificed 20 days later. We observed that mice injected with untreated WM-266-4 cells developed more metastases than mice injected with WM-266-4 cells pre-treated with 1 or 5 µM atorvastatin (Figure 4A and illustrated by a representative lung photomicrograph with metastatic area lesions in Figure 4B). These results confirm that atorvastatin pre-treatment inhibited melanoma cell colonization of the lungs through metastasis.

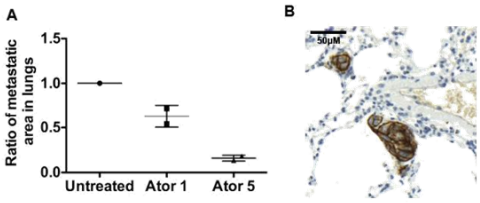

Figure 4: Atorvastatin pre-treatment reduces lung metastases development. 5×10^5 WM-266-4 cells untreated or pre-treated with Atorvastatin at 1 and 5 µM for 48 h (Ator) were injected intravenously into the tail vein of 8 NMRI nu/nu mice. Lung metastases were microscopically quantified and measured after IHC staining (A). Results are illustrated as ratio of metastatic area in lungs. A representative lung photomicrograph with metastatic areas lesions is shown (B).

Atorvastatin intraperitoneal injections inhibit subcutaneous melanoma growth

We next tested whether intraperitoneal injections of atorvastatin could reduce the growth of tumors *in vivo*. One day after subcutaneous implantation of 5×10^5 untreated WM-266-4 cells, mice were injected intraperitoneally with 0.1 ml PBS alone or 30 µg atorvastatin daily for 16 consecutive days. This dose of atorvastatin was chosen because it is comparable to that used in humans to treat hypercholesterolemia. We observed that these atorvastatin injections significantly slowed subcutaneous tumor growth (Figure 5). These results show that atorvastatin is a drug efficient *in vivo* on untreated tumor cells.

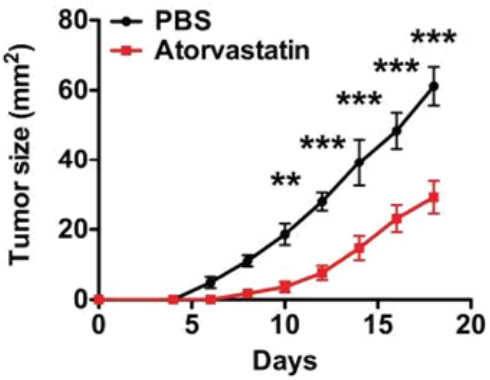

Figure 5: Daily intra-peritoneal injections of Atorvastatin reduce *in vivo* local untreated melanoma growth. 5×10^5 untreated WM-266-4 cells were injected subcutaneously into the flank of eleven NMRI nu/nu mice. Mice were then treated by daily IP injections with either PBS alone or with 30 µg (1 mg/kg) Atorvastatin. Local tumor sizes were measured serially. The results are expressed as mean size (mm^2) of tumors from group of nine mice each. Results are illustrated as mean ± SEM (error bars, n=2 experiments). $^{**}P<0.005$; $^{***}P<0.001$ versus control condition using the two-way ANOVA test.

Atorvastatin injections increase splenic NK cell concentration and activation

In an effort to determine the mechanism of action of our observed statin-induced control of melanoma development, we tested the capacity of atorvastatin intraperitoneal injections to increase NK cell activity. Nude mice, which only have innate immune effectors available for immunological rejection of tumor cells, were injected for 6 consecutive days with either 0.1 ml PBS alone or 30 µg atorvastatin. These injections induced a significant increase in splenic NK cell concentration and activation (Figures 6A and 6B).

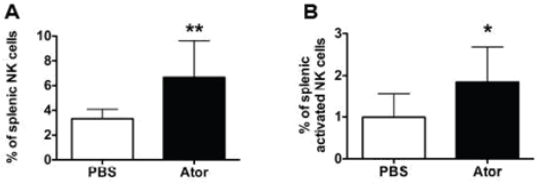

Figure 6: Daily intra-peritoneal injections of Atorvastatin increases splenic NK cells concentration and activation. Two groups of four nude mice were injected IP with 0.1 ml PBS alone or with 30 µg Atorvastatin for 6 consecutive days. Mice were sacrificed the following day. Splenic NK cells concentration was detected by flow cytometry with anti-NK1. 1-APC mAb (A) and activated NK cells with anti-CD69-PE and anti-NK1.1-APC mAb (B). Results are expressed as % of splenic cells (error bars, n=2 experiments). $^{*}P<0.05$ and $^{**}P<0.005$ versus control condition (PBS) using t-test.

Together our data suggest that intraperitoneal atorvastatin injections favored an innate anti-melanoma immune response via two linked mechanisms: an increase in activated NK cells and a tumoral overexpression of the NKG2D ligand MICA, which together facilitated the killing of human melanoma cells by murine NK cells.

Discussion

This work follows our first study on the contribution of statins to the innate anti-melanoma immune response [6]. The WM-266-4 melanoma cell line chosen for the present study is useful because it induces easily identifiable and quantifiable lung metastases after intravenous injection. With this tumor cell line we were able to confirm that statin treatment induces the overexpression of membrane-expressed MICA. WM-266-4 melanoma cells have been treated by two concentrations of atorvastatin (1 and 5 µM) previously describe by our group to promote, on melanoma cell surface, the over expression of different of immunological markers promoting anti melanoma immune response [3,5,6].

Our previous results, using two other melanoma cell lines LB1319-MEL and BB74-MEL, shown that pretreatment of these tumor cells with Atorvastatin at 5 µM for 48h induce a strong MICA overexpression on tumor cells membrane, enhance NK cell anti-tumor response and consequently inhibit local melanoma growth and pulmonary metastases development [6]. MICA is expressed on tumors or stressed cells and is a ligand for the activating receptor NKG2D. This receptor, which recognizes both human and murine tumor cells, plays an important role in the immune control of cancers [22].

Using WM-266-4 melanoma cells, we show that the pre-treatment of tumor cells with atorvastatin before injection into nude mice induced both a shrinkage of local tumor growth and a reduction in lung metastases. Above all, we demonstrate for the first time that statins, and in particular atorvastatin, may be of interest for the immunotherapy of melanoma. Moreover inhibition of RhoA/ROCK pathway is also induced by Atorvastatin treatment at 1 and 5 µM, indeed the statins block the mevalonate pathway leading to decrease isoprenylation of Ras and Rho GTPases, which blocks their activity. Our previous papers have shown that inhibition of RhoA/ROCK activity favors anti-melanoma immune response through MHC-class I, costimulatory molecules and FasL overexpression on melanoma cells membrane [3-5]. Furthermore, as previously described, the inhibition of ROCK activity reduces melanoma cells migration, invasion and metastases [23-27]. Consequently Atorvastatin pre-treatment of WM-266-4 cells at 1 µM mainly reduces local tumor growth and metastasis development through MICA overexpression and NK cell activity. But other mechanisms are probably also involved namely Atorvastatin-induced FasL overexpression and inhibition of melanoma cells invasion and metastasis, which is induced by ROCK inhibition. These latter two mechanisms probably play major roles in the nude mice injected with WM-266-4 cells pre-treated with Atorvastatin at 5 µM.

Indeed, the local growth of tumors obtained from the injection of untreated WM-266-4 cells was significantly slower when mice were injected daily with atorvastatin at a dose equivalent to human treatment for cardiovascular problems. Moreover, these injections induced an increase in the presence of activated NK cells in the spleens of injected mice. This is important because since activated NK cells express NKG2D receptors they efficiently kill MICA-positive melanoma cells. Conversely, it has previously been shown in Beige SCID mice, which do not have functional NK cells, that oral treatment with atorvastatin does not significantly reduce subcutaneous melanoma development [17]. These data strongly suggest that in our experimental nude mouse model, the atorvastatin-induced control of subcutaneous melanoma growth is dependent on NK cell activity. Moreover, previous studies of the anti-inflammatory effects of atorvastatin [23,24] suggest that in our experiments the atorvastatin-induced increase in activated ($CD69^+$) NK cells is not linked to a direct effect of atorvastatin on NK cells but is more likely due to atorvastatin-induced MICA overexpression in melanoma cells. However, intraperitoneal statin injections induce systemic disruptions, which could also interfere with the innate anti-melanoma immune response by acting on Tγδ cells.

Moreover, several cellular functions involving GTPases of the Ras family are likely to be disrupted by these statin injections. Interestingly Collisson et al. showed that, independently of the immune response, and at plasma concentrations similar to those used to treat hypercholesterolemia, atorvastatin inhibited the *in vivo* metastasis of melanoma cells overexpressing RhoC [17]. In our previous work we have shown that inhibitors of the RhoA GTPase favor an adaptive anti-melanoma immune response [3-5], and more recently we have described a beneficial role of statins in favoring the innate component of this immune response [6]. The present paper reinforces the idea that statins, and in particular atorvastatin, could be promising drugs for melanoma therapy.

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