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Atorvastatin inhibited Rho-associated kinase 1 (ROCK1) and focal adhesion kinase (FAK) mediated adhesion and differentiation of $CD133^+CD44^+$ prostate cancer stem cells

Satyanarayana Rentala [a,*], Ramakrishna Chintala [b], Manohar Guda [c], Madhuri Chintala [d], Aruna Lakshmi Komarraju [a], Lakshmi Narasu Mangamoori [e]

[a] Department of Biotechnology, GITAM University, Visakhapatnam, Andhra Pradesh, India
[b] GITAM Institute of Science, GITAM University, Visakhapatnam, Andhra Pradesh, India
[c] Department of Urology, King George Hospital, Visakhapatnam, Andhra Pradesh, India
[d] Department of Obstetrics and Gynaecology, King George Hospital, Visakhapatnam, Andhra Pradesh, India
[e] Centre for Biotechnology, IST, Jawaharlal Nehru Technological University, Hyderabad, Andhra Pradesh, India

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ABSTRACT

Prostate cancer has become a global health concern and is one of the leading causes of cancer death of men after lung and gastric cancers. It has been suggested that the 3-hydroxy-3-methyl-glutarylcoenzyme-CoA (HMG-CoA) reductase inhibitor atorvastatin shows anticancer activity in prostate cancer cell lines. To this end, we analyzed the influence of atorvastatin on the cell adhesion and differentiation of $CD133^+CD44^+$ cells derived from prostate cancer biopsies and peripheral blood. $CD133^+CD44^+$ cells were treated with atorvastatin (16–64 μM) for different time periods. Cell adhesion to endothelial cell monolayers and differentiation into prostate cancer cells were evaluated. α1, β1 and α2β1 integrins adhesion receptors and the downstream target of atorvastatin Rho-dependent kinase (ROCK) and focal adhesion kinase (FAK) were analyzed by Western blot. Further blocking studies with the ROCK inhibitor H1152, anti-FAK antibody and anti-integrin α1 and β1 antibodies were carried out. Atorvastatin treatment inhibited dose-dependently cell attachment to endothelium and differentiation. The inhibitory effect of atorvastatin on cell adhesion was associated with decreased expression of integrins α1 and β1 and phosphorylated MYPT1 and FAK. Furthermore, atorvastatin strongly reduced ROCK1 and FAK mediated differentiation of $CD133^+CD44^+$ cells, which was confirmed by antibody treatment. Atorvastatin modified the expression of cell adhesion molecules and differentiation markers. These beneficial effects of atorvastatin may be mediated by ROCK and FAK signaling pathway. The data presented may point to novel treatment options for prostate cancer.

1. Introduction

Atorvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is a widely used and well tolerated drug for treating hypercholesterolemia, coronary heart disease and stroke [1]. Mevalonate biosynthesis is catalyzed by HMG-CoA reductase [1]. Statins reduce the synthesis of mevalonate by inhibiting HMG-CoA reducatse finally leading to the blockade of Rho GTPases with their effector proteins ROCK [2]. Since ROCK is involved in cancer stem cells adhesion and homing, it is suggested that down-regulation of these proteins by atorvastatin may reduce cancer stem cell adhesion [3,4]. Indeed, novel reports demonstrate that atorvastatin treatment exerts further anticancer effects in several human malignancies, including breast, colon and prostate cancer [5,6].

Prostate cancer is a major public health concern in many industrialized countries. It is predominantly a disease of elderly men, with its incidence increasing steeply in the seventh decade of life. Recent studies have indicated that a subpopulation of cells, cancer stem cells (CSCs), is present in prostate cancer [7,8]. CSCs are capable of self-renewal and are responsible for tumor maintenance and metastasis [9–12]. Our previous reports have indicated that CSCs in prostate cancer tissues express CD133, MDR1, CD44, integrins α2β1, α1 and β1 [13]. On ligand binding, integrins transduce signals into the cell interior; they can also receive intracellular signals that regulate their ligand-binding affinity. Our previous reports suggest that α2β1, α1 and β1 integrins play major role in prostate cancer stem cell adhesion and differentiation.

* Corresponding author. Address: Department of Biotechnology (GIT), GITAM University, Visakhapatnam 530 045, Andhra Pradesh, India.
E-mail addresses: dr.rsn79@gmail.com, rsn@gitam.edu (S. Rentala).

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In this paper, we analyzed the cell adhesion and differentiation of $CD133^+CD44^+$ cells after treating with atorvastatin in. The expression pattern of integrin subunits was further evaluated by Western blot and correlated with cancer stem cell adhesion and differentiation. We concluded that atorvastatin inhibits the differentiation of prostate cancer stem cells by modulating ROCK1 and FAK signaling pathway. Taken together, these data suggest that atorvastatin may provide a therapeutic advantage for prostate cancer treatment but this requires further evaluation.

2. Materials and methods

All normal and prostate cancer tissues were obtained from volunteers in Sri Krishna City Hospital, Andhra Pradesh, India. The study was approved by the Institutional Ethics Committee/Institutional Review Board of Sri Krishna City Hospital.

2.1. Isolation of $CD133^+$ cells from prostate tissue biopsy specimens

$CD133^+$ cells were isolated from cancer patients (suffering from adeno-carcinoma of prostate – whose prostate-specific antigen levels are >4 ng mL^{-1}) who were 60–70 years of age. Biopsy samples ($n = 7$) were digested with a trypsin-collagenase mixture (Sigma, USA) to dissociate the cells. The $CD133^+$ cells were selected using magnetic beads and analyzed for the presence of CD133 (AC133; Miltenyi Biotech, Singapore). The $CD133^+$ cells were double stained with CD44 and sorted for $CD133^+CD44^+$ cells using magnetic cell sorter. The purity of $CD133^+CD44^+$ cells in the sorted samples was routinely more than 97%. The immunolabelling of CD133 was performed in prostate cancer tissues using a monoclonal CD133/1 antibody (AC133, Miltenyi Biotech) conjugated to biotin, and avidin-fluorescein isothiocyanate (FITC) was used to detect the biotin-CD133/1 antibody. The expression profile of CD133 and CD44 was studied by flow cytometry using a FITC-labeled rabbit antihuman CD44 antibody (Sigma, USA).

2.2. Isolation of $CD133^+$ cells from peripheral blood samples

Mononuclear cells were isolated from the blood samples of prostate cancer patients ($n = 7$) using a Ficoll gradient (Biocoll 1077; Sigma, USA). The cells were selected for CD133 expression on a magnetic column using magnetic beads and were then tested for the presence of CD133 (AC133). The $CD133^+$ cells were double stained with CD44 and sorted for $CD133^+CD44^+$ cells using magnetic cell sorter. The purity of $CD133^+CD44^+$ cells in the sorted samples was routinely more than 97%. The expression profile of CD133 and CD44 was studied by flow cytometry using a FITC-labeled rabbit antihuman CD44 antibody (Sigma, USA).

2.3. Culturing prostate tissue-derived and peripheral blood-derived $CD133^+CD44^+$ cells

The $CD133^+CD44^+$ cells that were derived from peripheral blood and from the prostate tissues of prostate cancer patients were cultured on plates coated with fibronectin (50 μg mL^{-1}) and laminin (50 μg mL^{-1}) in Iscove's modified Dulbecco's medium containing 10% Fetal calf serum, bovine transferrin (200 μg mL^{-1}), 100 IU/mL penicillin and 100 μg/mL streptomycin and 20 mM HEPES buffer (Sigma, USA), hydrocortisone (2 μmol L^{-1}), hepatocyte growth factor (HGF) (20 ng mL^{-1}), and GM-CSF (granulocyte-monocyte colony- stimulating factor) (20 ng mL^{-1}) (all from PeproTech Asia, Israel). Cells were cultured for 7–10 days, at which point half of the medium was replaced with fresh medium every other day. The cultured cells were trypsinized and analyzed for the expression profiles of α1, β1 and α2β1 integrins using an FITC-labeled rabbit anti-human antibodies and a rabbit anti-human β-actin antibody (all are from Sigma, USA).

2.4. Monolayer adhesion assay

To investigate adhesion of $CD133^+CD44^+$ cells to endothelial cells, human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins and harvested by enzymatic treatment with dispase (Sigma, USA). HUVECs were maintained in Medium 199 (Invitrogen, India) supplemented with 10% FCS, 20 μg/mL vascular endothelial cell growth factor (Roche, Germany), 5 U/mL heparin (Roche), 100 ng/mL gentamycin (Invitrogen, India) and 20 mM HEPES (Sigma, USA). HUVECs were grown in a humidified 5% CO2 incubator at 37 °C. The purity of isolated HUVEC cultures was controlled by staining with fluorescein isothiocyanate (FITC)-labeled monoclonal antibody against CD31 (PECAM1) (Pharmingen, USA). To analyze $CD133^+CD44^+$ cells adhesion, HUVECs were transferred to 6-well multiplates in complete HUVEC medium. When a confluence of about 80% was reached, $CD133^+CD44^+$ cells were detached from the culture flasks and 0.5×10^6 cells were carefully added to the HUVEC monolayer for 60 min. Subsequently, non-adherent $CD133^+CD44^+$ cells were washed off using warmed (37 °C) Iscove's modified Dulbecco's medium. The remaining cells were fixed with 1% glutaraldehyde. Adherent cells were counted in five different fields of a defined size (5×0.25 mm^2) using a phase contrast microscope (×20 objective) and the mean cellular adhesion rate was calculated.

2.5. Differentiation of prostate tissue-derived and peripheral blood-derived $CD133^+CD44^+$ cells

To investigate the role of ROCK1 and FAK during differentiation of $CD133^+CD44^+$ cells that were derived from the prostate tissues and peripheral blood of prostate cancer patients, cells were cultured on plates coated with fibronectin (50 μg mL^{-1}) and laminin (50 μg mL^{-1}) in Iscove's modified Dulbecco's medium as given in the methodology. In addition to the previously mentioned growth factors, insulin-like growth factor (IGF) (20 ng mL^{-1}), basic fibroblast growth factor (bFGF) (20 ng mL^{-1}), epidermal growth factor (EGF) (20 ng mL^{-1}) (all from PeproTech Asia, Israel) were used as differentiation inducers. Cells were cultured in serum-free basal medium for 7–10 days, at which point half of the medium was replaced with fresh medium every other day. The cultured cells were trypsinized and analyzed for the expression profiles of the androgen receptor, prostate specific antigen, CD57, human glandular kallikrein 2 (hK2), PLA2G7 and β-actin were studied using Western blot and flow cytometry.

2.6. Drug and antibody treatment

Atorvastatin (Sigma, USA) and activated prior to the experiments by alkaline hydrolysis of the lactone moiety according to the manufacturer's protocol. $CD133^+CD44^+$ cells were treated for 24, 48 and 72 h with various concentrations of atorvastatin (0–64 μM) or with vehicle with fresh changes of culture medium and atorvastatin after 48 h. In additional experiments, mevalonate (3.2 mM; Sigma, USA) was added to the medium containing atorvastatin to address the atorvastatin site of action along the mevalonate pathway. To confirm the involvement of specific pathways, the highly selective Rho-kinase inhibitor H1152 (1 μM; EMDMillipore, India) was added to the medium instead of Atorvastatin. In some experiments, functional analysis of adhesion and differentiation was done using rabbit anti-human α1 (3 μg mL^{-1}), β1 (3 μg mL^{-1}), ROCK1 (5 μg mL^{-1}) and FAK (5 μg mL^{-1}) antibodies to block α1, β1, ROCK1 and FAK (pTyr397) antigens in cell culture.

2.7. Western blot

Total integrin α1 (CD49a), integrin β1 (CD29), integrin α2β1 (CD49b,CD29), FAK, phosphorylated FAK, ROCK1, MYPT and phosphorylated MYPT content in $CD133^+CD44^+$ cells was evaluated by Western blot analysis using mouse anti-CD29, anti-human CD49a, CD49b, rabbit anti-human FAK, rabbit anti-human phosphorylated FAK, mouse anti-ROCK1 (all from BD Pharmingen, USA), goat anti-MYPT1 and anti-p-MYPT1 (Thr 696; Santa Cruz Biotechnology, USA). The differentiation markers prostate specific antigen, prostate specific membrane antigen and human glandular kallikrein 2 (hK2) were evaluated by Western blot using anti-human prostate specific antigen, anti-human prostate specific membrane antigen and anti-human glandular kallikrein 2 (hK2) $CD133^+CD44^+$ cell lysates (50 μg protein) were separated by electrophoresis on 7% polyacrylamide SDS gels and transferred to nitrocellulose membranes. Determination of β-actin with anti-β-actin antibody (Sigma, USA) served as loading control. Blots were blocked (10% non-fat dry milk in 1 mM Tris, 150 mM NaCl, pH 7.4) for 1 h, incubated 1 h at room temperature with primary antibody (diluted according to manufacturer's instructions) and then incubated for 1 h with horseradish peroxidise conjugated secondary antibody (Pharmingen, USA) diluted 1:1000. Proteins were detected with ECL™ Western blot detection reagents (GE Healthcare, Germany) and visualized after exposure to X-ray film.

2.8. Flow cytometry

A total of 5×10^5 cells out of freshly isolated cells from patient biopsies and peripheral blood samples were stained with corresponding primary and secondary antibodies. The primary antibodies used in the study were biotin-labeled CD133 (Miltenyi Biotech), biotin-labeled CD44 (Miltenyi Biotech), FITC-labeled rabbit anti-human CD57 clone NK-1, and FITC-labeled rabbit anti-human androgen receptor (all from Pharmingen). The secondary antibodies used in this study were anti-biotin-PE and streptavidin-FITC (all procured from Pharmingen). Cells were analyzed on a customized BD FACScan (Becton–Dickinson, Singapore) using filters of 530/28 BP for FITC and 575/26 BP for PE and a 488-nm argon laser to excite both PE and FITC.

2.9. Statistical analysis

Differences between groups were determined by one-way ANOVA followed by Dunnett's *t*-test using GraphPad InStat version 3.06 (GraphPad software Inc., San Diego, CA, USA). All *P*-values <0.05 were considered to be statistically significant.

3. Results

3.1. Isolation and characterization of prostate tissue derived $CD133^+$ cells

The tissues from both normal and prostate cancer patients were sectioned and stained with hematoxyline and eosin (H-E) staining. Microscopic examination of these two sections revealed a clear distinction between the two tissues. Sections of normal prostatic tissues as shown in Fig. 1A indicate a normal morphology whereas prostate cancer tissue sections were shown in Fig. 1B indicate cell mass clumping with extensive invasion of blood vessels into the tissue that is typical of adeno-carcinoma with metastasis. The immune-histochemistry of CD133 in prostate cancer tissues, stained with FITC (green) was shown in Fig. 1D, corresponding control-IgG labeling was shown in Fig. 1C. Nuclei were labeled with DAPI (blue). $CD133^+CD44^+$ cells were isolated from prostate tissue samples of seven prostate cancer patients. To characterize $CD133^{+-}$ $CD44^+$ cells in prostate cancer tissues, flow cytometry analysis was used to detect the expression profile of prostate cancer stem cell markers. The analysis revealed a high percentage (97%) of $CD133^+CD44^+$ cells were isolated from the prostate cancer tissues as shown in Fig. 1F. Isotype controls IgG-FITC and IgG-PE were shown in Fig. 1E.

3.2. Atorvastatin alters prostate cancer stem cell adhesion to HUVECs

$CD133^+CD44^+$ cells were treated with 16, 32 and 64 μM atorvastatin for 24, 48 and 72 h to study the effect of atorvastatin on adhesion to HUVECs. Fig. 2A and B show the adhesion kinetics of treated versus non-treated cells. Atorvastatin blocked significantly the adhesion of $CD133^+CD44^+$ prostate cancer stem cells to endothelium when it was applied for 48 or 72 h in all doses. A 24-h application of 16 μM atorvastatin did not prevent prostate cancer tissues derived $CD133^+CD44^+$ cells attachment to HUVECs, whereas peripheral blood derived $CD133^+CD44^+$ cells adhesion to endothelium was markedly decreased compared to control cells. Generally, $CD133^+CD44^+$ cells derived from prostate cancer tissues and peripheral blood were more sensitive to high doses (32 and 64 μM) of atorvastatin compared to the 16 μM atorvastatin application. To evaluate the effect and specificity of the inhibitory potential of atorvastatin, cells were pre-treated with mevalonate in combination with atorvastatin. This study was performed with the atorvastatin dose of 32 μM with a pre-treatment duration of 48 h. Atorvastatin reduced markedly $CD133^+CD44^+$ cells adhesion to endothelial cells. More cells were attached to HUVECs when atorvastatin was applied in combination with mevalonate (Fig. 2C and D), although mevalonate alone did not influence the adhesion characteristics of $CD133^+CD44^+$ cells (data not shown). To confirm that the effect of atorvastatin was mediated through Rho/Rho-kinases, we used the ROCK inhibitor H1152, which largely diminished the attachment rates of $CD133^+CD44^+$ cells to levels comparable to those induced by atorvastatin. Since $CD133^+CD44^+$ cells derived from prostate cancer tissues and peripheral blood showed comparable adhesion and growth characteristics, further studies concentrated on atorvastatin doses of 32 and 64 μM.

3.3. Atorvastatin modulates the integrin expression pattern

α1 and β1 integrins were expressed in $CD133^+CD44^+$ cells derived from prostate cancer tissues and peripheral blood and whose expressions were decreased when atorvastatin was applied for 48 or 72 h. α2β1 expression was also diminished in $CD133^+CD44^+$ cells derived from prostate cancer tissues and peripheral blood after atorvastatin application (Fig. 2E and F).

Functionality of integrins α1 and β1 and their signaling molecules ROCK1 and FAK $CD133^+CD44^+$ cells derived from prostate cancer tissues and peripheral blood were pre-incubated with antibodies against α1 and β1 integrins and integrin signaling molecules ROCK1 and FAK and subsequently allowed to attach to endothelial monolayer. The adhesion rates of $CD133^+CD44^+$ cells derived from prostate cancer tissues and peripheral blood significantly declined following α1 and β1 and signaling molecules ROCK1 and FAK receptors blockade as compared with untreated controls (Fig. 3A and B). The adhesion pattern of atorvastatin (64 μM) pre-treatment in $CD133^+CD44^+$ cells derived from prostate cancer tissues (Fig. 3A) and peripheral blood (Fig. 3B) was declined and the effect was similar to that of combined blockade of ROCK1 and FAK antigens.

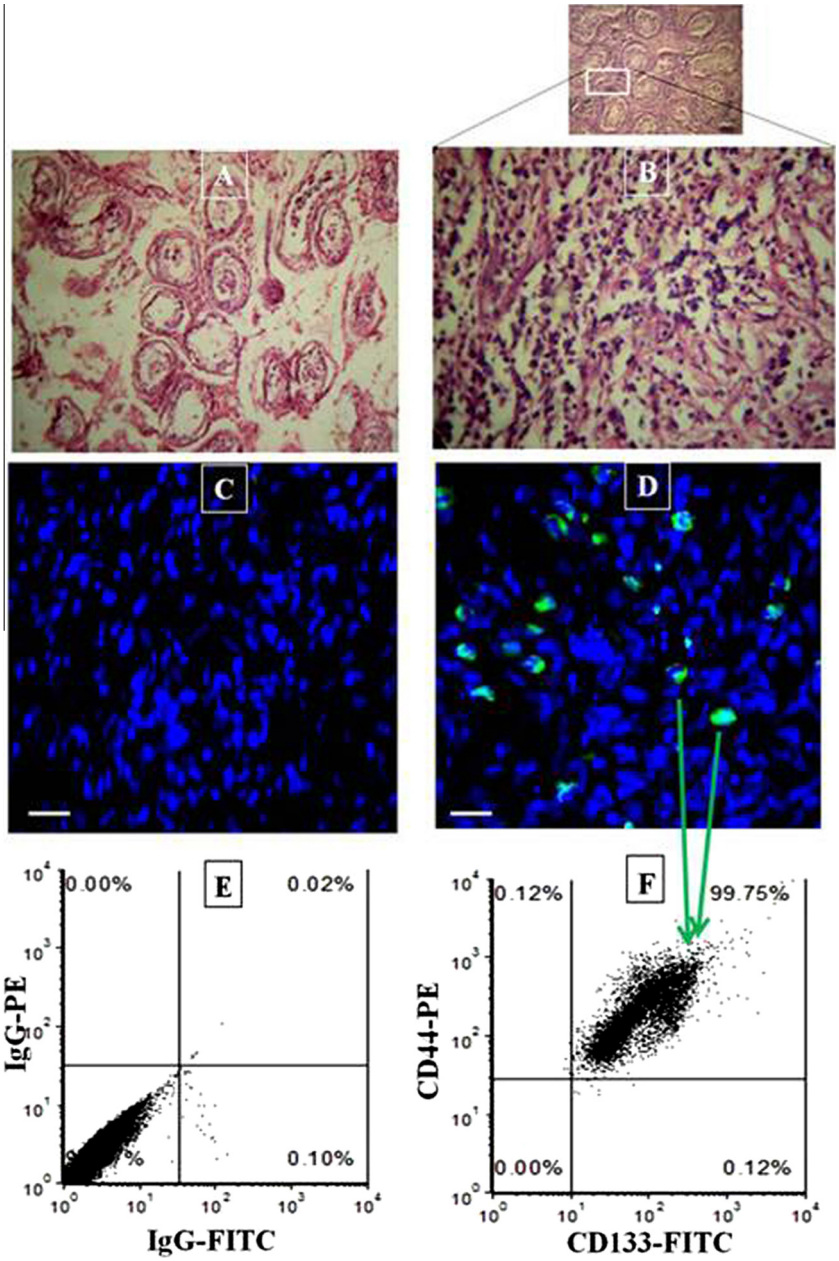

Fig. 1. Isolation of $CD133^{+}CD44^{+}$ cells from adeno-carcinoma of prostate tissues. Hemotoxylin–eosin staining of normal prostate tissue (A) and adeno-carcinoma of prostate tissue (B) were shown. The immunolabelling of CD133 in prostate cancer tissues, which was stained with FITC (green) was shown in (D), corresponding control-IgG labeling was shown in (C). Nuclei were labeled with DAPI (blue). Scale bars = 50 μm. (E) Represents flow cytometry report of isotype control IgG-FITC and isotype control IgG-PE. (F) Represents flow cytometry report of CD133-FITC and CD44-PE. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Atorvastatin modifies the ROCK1 and FAK activities

The expression status of Rho/Rho-kinase and myosin phosphatase targeting subunit, p-Thr^{696}-MYPT1, a marker for Rho-kinase activity and phosphorylated FAK ($pTyr^{397}$-FAK) were determined. Western blot analysis for ROCK1 and FAK showed that both were suppressed by atorvastatin treatment. The ROCK1 and FAK activity pattern of atorvastatin pre-treatment in $CD133^{+}CD44^{+}$ cells derived from prostate cancer tissues (Fig. 3C) and peripheral blood (Fig. 3D). Additionally, atorvastatin diminished Rho-kinase and FAK activities as demonstrated by reduced phosphorylation of MYPT and FAK and induced significant reduction of α1, β1 and α2β1 integrin expression.

3.5. Atorvastatin modulates ROCK1 and FAK signaling differentiation of $CD133^{+}CD44^{+}$ prostate cancer stem cells

ROCK1 and FAK mediated signaling during differentiation of several types of stem cells was already well studied. To investigate the role of atorvastatin in ROCK1 and FAK mediated differentiation of $CD133^{+}CD44^{+}$ cells derived from prostate cancer tissues and peripheral blood, antibodies against α1 and β1 integrins and signaling molecules ROCK1 and FAK and atorvastatin (64 μM) were used in the cell culture for differentiation. The differentiation of $CD133^{+}CD44^{+}$ derived from prostate cancer tissues (Fig. 4A and C) and peripheral blood (Fig. 4B and D) cells into CD57 and androgen receptor (AR) positive cells (flow cytometry data) and PLA2G7,

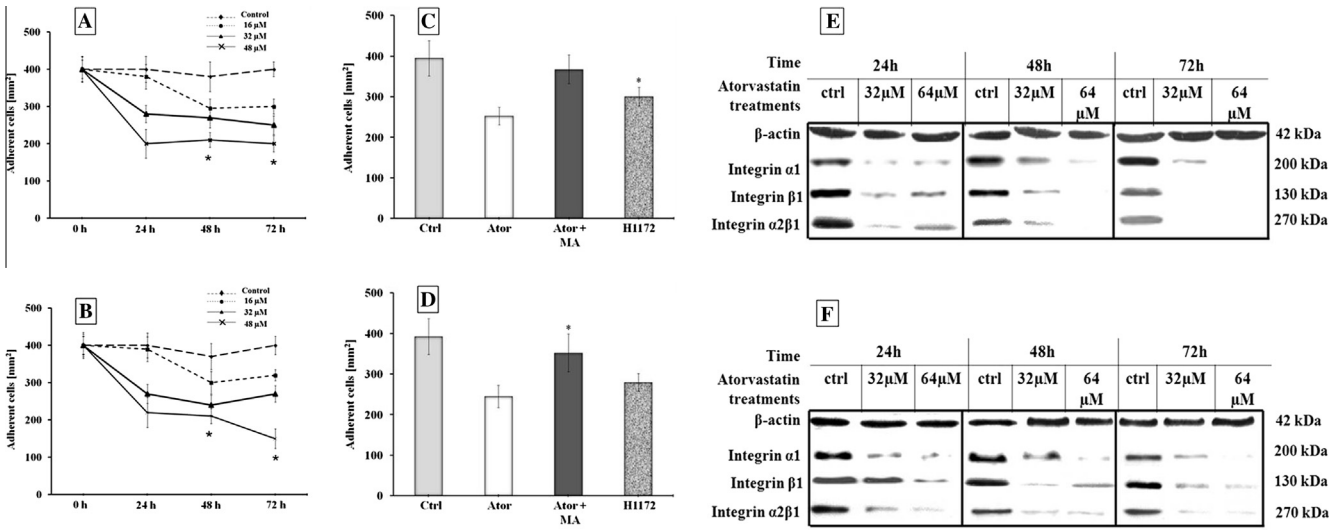

Fig. 2. Atorvastatin regulates adhesion of $CD133^+CD44^+$ prostate cancer stem cells via ROCK1 and FAK signaling pathway. $CD133^+CD44^+$ cells were treated with different doses of atorvastatin (0–64 μM) for 24, 48 and 72 h or remained untreated (ctrl). Adhesion of $CD133^+CD44^+$ cells derived from prostate cancer tissues to HUVECs was shown in (A) and of $CD133^+CD44^+$ cells derived from peripheral blood to HUVECs was shown in (B). Adhesion of $CD133^+CD44^+$ cells derived from prostate cancer tissues to HUVECs in presence of atorvastatin, mevalonate and H1152 was shown in (C) and of $CD133^+CD44^+$ cells derived from peripheral blood to HUVECs was shown in (D). Ctrl = control, Ator = atorvastatin (32 μM), MA = mevalonate (3.2 mM) and H1152 (1 μM) = ROCK1 inhibitor. α1, β1 and α2β1 integrin expression of $CD133^+CD44^+$ cells derived from prostate cancer tissues was shown in (E) and of $CD133^+CD44^+$ cells derived from peripheral blood was shown in (F). Data are given as the mean ± SEM (*$p < 0.05$ vs. ctrl). β-actin served as internal loading control.

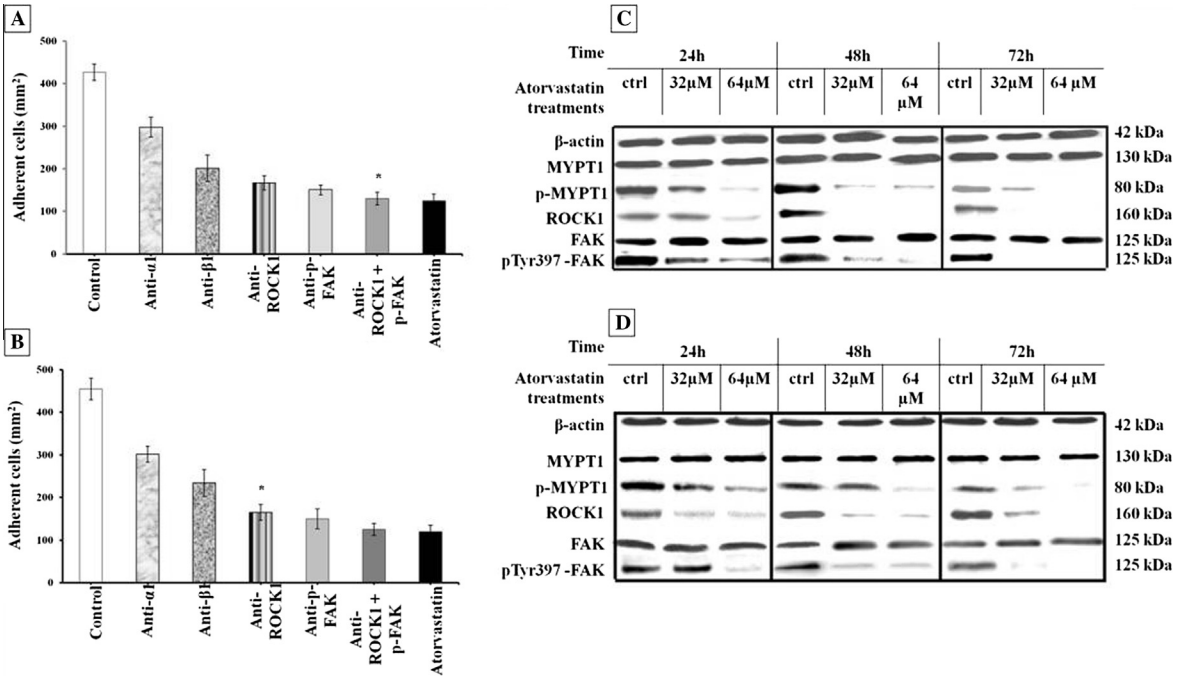

Fig. 3. Atorvastatin inhibited adhesion of prostate cancer stem cells through integrins in ROCK1 and FAK signaling pathway. Integrins α1 and β1, ROCK1 and/or FAK were blocked by monoclonal antibodies and atovastatin was treated. Adhesion of $CD133^+CD44^+$ cells derived from prostate cancer tissues to HUVECs was shown in (A) and of $CD133^+CD44^+$ cells derived from peripheral blood to HUVECs was shown in (B). Expression profile of ROCK-I, MYPT, phosphorylated-MYPT, FAK and phosphorylated-FAK of prostate cancer tissues derived $CD133^+CD44^+$ cells was shown in (C) and of peripheral blood derived $CD133^+CD44^+$ cells was shown in (D). Data are given as the mean ± SEM (*$p < 0.05$ vs. ctrl). β-actin served as internal loading control.

prostate specific antigen and human glandular kallikrein 2 (hk2) expression (Western blot data) was decreased upon the treatments. The inhibition of the differentiation of $CD133^+CD44^+$ derived from prostate cancer tissues and peripheral blood upon atorvastatin treatment was significantly more than any other treatments.

4. Discussion

The present study showed that atorvastatin effectively downregulated adhesion and differentiation of $CD133^+CD44^+$ cells derived from prostate cancer tissues and peripheral blood and that these effects were associated with the decreased expression of

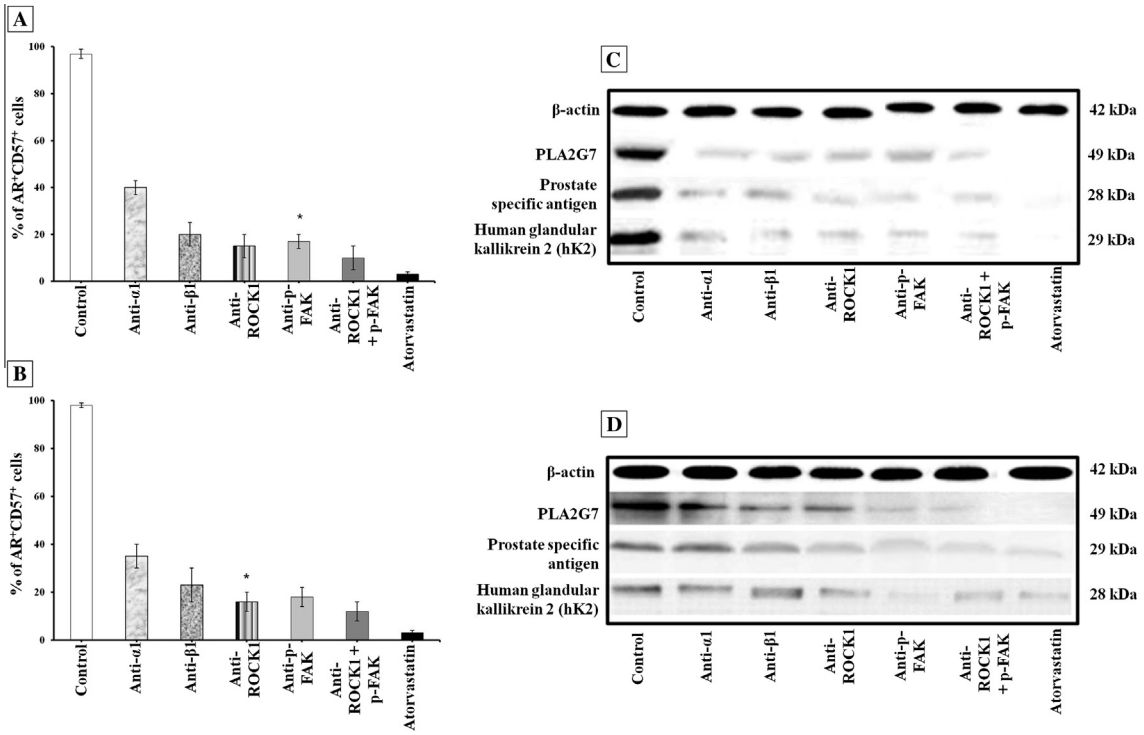

Fig. 4. Atorvastatin inhibited differentiation of $CD133^+CD44^+$cells through ROCK1 and FAK signaling pathway. Integrins α1 and β1, antigens ROCK1 and FAK were blocked with monoclonal antibodies and atorvastatin was treated. Differentiation of $CD133^+CD44^+$ cells derived from prostate cancer tissues to HUVECs was shown in Fig. 3A and of $CD133^+CD44^+$ cells derived from peripheral blood to HUVECs was shown in Fig. 3B. Expression profile of PLA2G7, prostate specific antigen and human glandular kallikrein 2 (hK2) of during differentiation of prostate cancer tissues derived $CD133^+CD44^+$ cells was shown in Fig. 3C and of peripheral blood derived $CD133^+CD44^+$ cells was shown in Fig. 3D. Data are given as the mean ± SEM ($^*p < 0.05$ vs. ctrl). β-actin served as internal loading control.

α1, β1, and α2β1 integrins. These changes were in parallel to the level of relative protein expression of ROCK1 and FAK and their activity as shown by reduced phosphorylation, suggesting that the activation of ROCK1 and FAK are involved in the carcinogenic processes in prostate cancer [14]. The adhesion of prostate cancer stem cells to vascular endothelium, subsequent disruption of the basement membrane, differentiation into tumor cells and invasion of tumor cells into the host tissue are crucial steps in metastasis of cancer cells [14]. Statins have been described to alter numerous cellular mechanisms. *In vitro* and *in vivo* studies showed that statins inhibit tumor cell growth, induce apoptosis, inhibit angiogenesis and impair the metastatic process [15,16]. Although simvastatin has been demonstrated to modulate the pathogenesis of many cancer types by inhibiting tumor cell growth dynamics, data on atorvastatin induced effects on prostate cancer stem cells are sparse [15,17,18]. The present *in vitro* model reveals that atorvastatin significantly influenced both the cell adhesion and differentiation behavior of prostate cancer stem cells.

In our previous studies, we reported that FAK plays a crucial role in adhesion and differentiation of prostate cancer stem cells [13]. Our study also provides evidence that prostate cancer can originate from CD133-enriched cells that express α1 and β1 integrins, which are crucial for the regulation of cellular adhesion to ECM and cellular differentiation.

To analyze the underlying biological mechanisms in atorvastatin-induced prostate cancer stem cell integrin receptor expression was evaluated, since integrins play a decisive role in tumor metastasis by mediating tumor cell targeting, arrest, adhesion and migration [4,19,20]. Integrin α1, β1 and α2β1 subunits have been reported to be expressed on the surface of prostate cancer stem cells [7,10,11,13]. In the current study, atorvastatin reduced α1, β1 and α2β1 expression. Furthermore, blocking studies using the functional anti-integrin α1 and β1 and anti-ROCK1 and anti-FAK antibodies resulted in strongly decreased adhesion of prostate cancer stem cells to HUVECs revealing the functional impact of integrins on adhesion of prostate cancer stem cells. But adhesion pattern of atorvastatin (64 μM) pre-treatment in $CD133^+$ $CD44^+$ cells derived from prostate cancer tissues (Fig. 3A) and peripheral blood (Fig. 3B) was similar to that of combined blockade of ROCK1 and FAK antigens, which clearly shows that atorvastatin inhibits the adhesion of prostate cancer stem cells. This effect may be due to down-regulation of ROCK1 and FAK signaling molecules.

Differentiation of $CD133^+CD44^+$ cells was found to be inhibited in the presence of anti-integrin α1 and β1 and anti-ROCK1 and anti-FAK blocking antibodies. Flow cytometry and Western blot analysis indicated that the expression of the androgen receptor, prostate-specific antigen, PLA2G7, CD57 and human glandular kallikrein (hk2) were inhibited in the presence of blocking antibodies [15]. But this differentiation pattern of atorvastatin (64 μM) pre-treatment in $CD133^+CD44^+$ cells derived from prostate cancer tissues (Fig. 4A and C) and peripheral blood (Fig. 4B and D) was similar to that of combined blockade of ROCK1 and FAK antigens, which clearly shows that atorvastatin inhibits the differentiation of prostate cancer stem cells. This effect may be due to down-regulation of ROCK1 and FAK signaling molecules. ROCK1 and FAK have important effects on cell fate. It is well established that RhoA/ROCK1 and FAK have the potential to regulate stem cell adhesion and differentiation [19]. Our study demonstrates that atorvastatin inhibits ROCK1 and FAK expressions as well as their activities, accompanied by reduced prostate cancer stem cell adhesion and differentiation (Figs. 2–4).

In conclusion, ROCK1 and FAK activation plays an important role in the carcinogenesis of prostate cancer, since their inhibition by atorvastatin may be associated with reduced adhesion to endothelium and integrin expression. Hence, our results demonstrate the anti-carcinogenic and beneficial role of atorvastatin in our

prostate cancer stem cell model and suggest that atorvastatin may represent an attractive candidate for therapeutic intervention. Thus, the most attractive application of statins will probably be as part of multidrug therapy.

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