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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

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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.

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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.

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