Simvastatin modulates β-catenin/MDR1 expression on spheres derived from CF41.Mg canine mammary carcinoma cells

P. Cruz1,2, F. Reyes1, C.G. Torres1

1 Laboratory of Biomedicine and Regenerative Medicine, Department of Clinical Sciences, Faculty of Veterinary and Animal Sciences, University of Chile, Santa Rosa Avenue 11735, La Pintana, 8820808 Chile
2 Ph.D program in Forestry, Agricultural and Veterinary Sciences, Faculty of Veterinary and Animal Sciences, University of Chile, Santa Rosa Avenue 11735, La Pintana, 8820808 Chile

Abstract

The presence of cancer stem-like cells (CSC) within canine mammary tumors, may explain partly local recurrence and spreading, since their ability to resist conventional antitumor treatments as chemo and radiotherapy. It has been recently described that simvastatin – a drug that inhibits synthesis of cholesterol – attenuates the proliferation of canine mammary CSC derived from CF41.Mg canine mammary carcinoma cells, promoting their chemosensitizing and apoptosis. The canonical Wnt/β-catenin pathway is usually activated at CSC and up-regulates multidrug resistance protein 1 (MDR1), triggering chemoresistance. In the present study, we analyze the effect of simvastatin on β-catenin/MDR1 expression in spheres obtained from the CF41.Mg cell line as a model of CSC. Simvastatin increased phosphorylation of β-catenin without affecting its total expression. Moreover, MDR1 expression was decreased by simvastatin. These results suggest that simvastatin would facilitate the degradation of β-catenin, decreasing MDR1 expression and contributing to the chemosensitizing effects of the statin on canine mammary CSC.

Key words: β-catenin, MDR1, simvastatin, canine mammary carcinoma cells, cancer stem cells

Introduction

Canine mammary cancer is a disease of frequent occurrence in veterinary medicine (Salas et al. 2015) and is considered a good biological model for human breast cancer (Pinho et al. 2012). Approximately 50% of canine mammary tumours are classified histologically as malignant, implying the potential to invade and/or metastasize to adjacent tissues. Options for treatment are limited, including surgery and chemotherapy (Salas et al. 2015). In this regard, a high proportion of all mammary cancer patients may suffer local recurrence or distant metastasis after surgery and chemotherapy, which appears closely correlated to poor chemosensitivity. Unfortunately, the molecular mechanisms involved in drug resistance are not yet fully understood (Klopfleisch et al. 2016).

Correspondence to: C.G. Torres, e-mail: crtorres@uchile.cl, tel.: +56 22 978 56 12
Cancer stem-like cells (CSC) are a subpopulation of tumour cells that exhibit several stemness properties such as auto-renewal, asymmetric cell division, and cellular plasticity. These cells have sphere-forming capacity, an ability that allows their in vitro isolation and further characterization. Since CSC display resistance to conventional treatments (chemo and radiotherapy) and high invasiveness, they may be responsible in part for tumour progression and metastasis (Pang and Ar-gyle 2015), which are characteristics of high histologi-cal grade in both canine and human mammary carcino-mas (Im et al. 2015). This ability would be acquired by a variety of mechanisms such as high expression of multidrug-resistance (MDR) transport-proteins associated with the excretion of xenobiotics, cell quiescence and arrest in the G0/G1 phase (Torres et al. 2015, Klopfeleisch et al. 2016).

One of the signaling pathways that are usually activated at CSC is the canonical Wnt/β-catenin pathway, which regulates many cellular functions including cell survival, migration, and differentiation. Once activated, β-catenin accumulates in the cytoplasm and translocates to the nucleus, upregulating target genes such as MDR1 (Flahaut et al. 2009). On the other hand, in the absence of Wnt, β-catenin is degraded by the Axin complex, by phosphorylating and inducing its ubiquitination and proteasomal degradation (Stamos and Weiss 2013). The MDR1 protein (ABCB1; p-glycoprotein/P-gp) is an ATP-binding cassette (ABC) transporter related to drug uptake and efflux, which is widely associated with multidrug resistance in several tumours including mammary cancer (Flahaut et al. 2009, Atil et al. 2016).

Many researchers have therefore been investigating novel drugs that target against some multidrug resistance molecules. The statins are a group of drugs mainly used to treat dyslipidemias, but which have shown promising pleiotropic effects against diverse types of cancer and other pathologies (Robin et al. 2014, Shen et al. 2015). Statins reduce serum cholesterol levels by competitively inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, preventing the synthesis of cholesterol. This inhibition causes a deficit of mevalonate, decreasing the formation of lipid isoprenoid intermediates involved in the posttranslational changes of several proteins, which explains many of the antitumour effects associated with statins (Kusama et al. 2006). Alternatively, it has been described that the effects of statins occur via other mechanisms, such as reduction in the expression of CD44, non-related with the Axin complex, by phosphorylating and inducing its ubiquitination and proteasomal degradation (Stamos and Weiss 2013). The MDR1 protein (ABCB1; p-glycoprotein/P-gp) is an ATP-binding cassette (ABC) transporter related to drug uptake and efflux, which is widely associated with multidrug resistance in several tumours including mammary cancer (Flahaut et al. 2009, Atil et al. 2016).

Drug. Simvastatin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was prepared in absolute ethanol (stock solution at 5 mM). Spheres-cells were incubated with 1 and 10 μM simvastatin for 48 h, and vehicle as control.

Western blotting. The proteins of interest were analyzed using western blot. After lysing statin-exposed and control cells with RIPA buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na,VO4, 1 mg/ml Leupetin, and protease inhibitors, these were sonicated and their total protein was quantified (BCA assay, Pierce). For electrophoresis, 30 μg of total protein was loaded in 10% polyacrylamide gels. Electrophoresis was run using appropriate chambers. Bands were then electro-transferred onto PVDF membranes, and immunodetected using appropriate primary antibodies (Table 1). The membranes were then incubated with anti-rabbit IgG (A6667) and anti-mouse IgG (A9917) peroxidase antibodies (1:5,000; Sigma-Aldrich; Merck KGaA) for 1 h at 4°C.
Table 1. Primary antibodies used for western blot analysis.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Clone</th>
<th>Source</th>
<th>Immunoglobulin subclass</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-catenin</td>
<td>14/β-catenin</td>
<td>Mouse</td>
<td>IgG₁</td>
<td>1:1000</td>
</tr>
<tr>
<td>Phospho-β-catenin</td>
<td>–</td>
<td>Rabbit</td>
<td>–</td>
<td>1:1000</td>
</tr>
<tr>
<td>P-glycoprotein</td>
<td>C219</td>
<td>Mouse</td>
<td>IgG₁</td>
<td>1:200</td>
</tr>
<tr>
<td>β-actin</td>
<td>mAbcam 8226</td>
<td>Mouse</td>
<td>IgG₁</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

* BD Biosciences Cat. 610154; * Cell Signaling Cat. 9561S; * Millipore Cat. 517310; * Abcam Cat. ab8226.

Statistical analysis. The Shapiro-Wilk test was used to determine normal distribution. ANOVA and Bonferroni tests were used to evaluate differences between samples. p<0.05 was considered significant. Data were analysed using Infostat Software for Windows.

Results

In spheres obtained from CF41.Mg cells, simvastatin did not affect total β-catenin expression in the studied time; however, statin significantly increased the phosphorylation of this protein, an effect that was concentration-dependent (p<0.05). On the other hand, MDR1 expression was significantly lower in response to 10 μM simvastatin at 48 h (p<0.05) (Fig. 1).

Discussion

Spheres derived from CF41.Mg cells exhibit stemness features such as chemoresistance to doxorubicin and paclitaxel, auto-renewal and high expression of CD44+/CD24-low phenotype (Torres et al. 2015). In this regard, the literature indicates that spheres from different canine mammary tumor cell lines exhibit common stemness characteristics as...
already described (Michishita et al. 2011, Rybicka and Krol 2016, Du et al. 2017). Therefore, CF41.Mg-
-spheres are a good model for studying CSC in re-
-sponse to several cytotoxic agents. Data shown here
indicate for the first time that simvastatin may be
blocking the signaling pathway β-catenin/MDR1,
which could partly explain the chemosensitizing effect
of this statin on canine mammary CSC previously de-
scribed (Torres et al. 2015). In that study, 10 μM sim-
vastatin sensitized CSC to the cytotoxic effect of 250
ng/ml doxorubicin, a MDR1 substrate, causing more
cell death than chemotherapeutic drug alone. This ef-
fect was observed at 72 h, an incubation time at which
simvastatin did not induce cytotoxicity alone.

Simvastatin induced an increase in the phos-
phorylation of β-catenin, an effect that promotes its
degradation and prevents its movement to the nu-
cleus. The antibody used here identifies phos-
phorylated β-catenin at serines 33, 37 and threonine
41, phosphorylated sites by glycogen synthase kinase
3β (GSK-3β), a kinase that is part of the Axin complex
(Stamos and Weiss 2013). This effect probably down-
regulates MDR1 gene transcription with a consequent
decrease in p-glycoprotein levels and the minor activ-
ity of this drug efflux pump. The effect of statins on
MDR1 expression has been previously described by
Siczkowski’s group, who verified that simvastatin de-
creased both expression and activity of this protein in
human neuroblastoma cells, which enhances the
proapoptotic effect of doxorubicin (Sieczkowski et al.
2010). These outcomes are consistent with that ob-
served by us.

CD44 is a surface glycoprotein highly expressed in
canine mammary CSC (Im et al. 2015), associated with
cell migration and cell-matrix interaction by
binding to hyaluronan, a major component of the ex-
tracellular matrix (Chanmee et al. 2016). In addition,
CD44 triggers activation of β-catenin, MDR1 and
Bcl-xL expression, inducing chemoresistance (Bour-
guignon et al. 2009). Since there is evidence that sim-
vastatin inhibits CD44 expression in human metastatic
breast cancer cells (Mandal et al. 2011), it would be
very interesting to explore the potential effects of sim-
vastatin on CD44 expression and drug resistance in
canine mammary CSC, to establish more precisely the
molecular mechanism by which this statin exerts its
chemosensitizing action.

These results suggest a role of simvastatin at the
level of the canonical pathway Wnt/β-catenin/MDR1.
This drug may represent a promising adjuvant thera-
peutic option against high grade canine mammary tum-
ours and with potential application in human breast
cancer; nevertheless, it is necessary to extend these re-
results to other mammary carcinoma cells and carry out
in vivo studies evaluating the findings described here.

Acknowledgements

This work was supported by Fondecyt grant
11110148 and the Office of Research and Develop-
ment, University of Chile. We thank Dr. Jose I. Arias
and Dr. Pablo F. Cespedes for their technical assist-
ance.

References

Atil B, Berger-Sieczkowski E, Bardy J, Werner M, Hoheneg-
ger M (2016) In vitro and in vivo downregulation of the
ATP binding cassette transporter B1 by the HMG-CoA
reductase inhibitor simvastatin. Naunyn Schmiedebergs
Arch Pharmacol 389: 17-32.

Bourguignon LY, Xia W, Wong G (2009) Hyaluronan-me-
diated CD44 interaction with p300 and SIRT1 regulates
beta-catenin signaling and NFkappaB-specific transcrip-
tion activity leading to MDR1 and Bcl-xL gene express-
ion and chemoresistance in breast tumor cells. J Biol
Chem 284: 2657-2671.

Chanmee T, Ontong P, Itano N (2016) Hyaluronan: a modu-
lator of the tumor microenvironment. Cancer Lett
375: 20-30.

Salinomycin inhibits canine mammary carcinoma in vitro

Flahaut M, Meier R, Coulon A, Nardou KA, Nigligi FK,
Martinet D, Beckmann JS, Joseph JM, M"uhlethaler-Mottet A, Gross N (2009) The Wnt recep-
tor FZD1 mediates chemoresistance in neuroblastoma
through activation of the Wnt/β-catenin pathway.
Oncogene 28: 2245-2256.

drug resistant breast cancer stem-like cells with combina-
tion of simvastatin and gamma-tocotrienol. Cancer Lett
328: 285-296.

Im KS, Jang YG, Shin JI, Kim NH, Lim HY, Lee SM, Kim
JH, Sur JH (2015) CD44+/CD24- cancer stem cells are
associated with higher grade of canine mammary carcino-
as. Vet Pathol 52: 1041-1044.

Klopflieisch R, Kohn B, Gruber AD (2016) Mechanisms of
tumor resistance against chemotherapeutic agents in vet-

Kusama T, Mukai M, Tatsuta M, Nakamura H, Inoue
M (2006) Inhibition of transendothelial migration and
invasion of human breast cancer cells by preventing ger-

Mandal CC, Ghosh-Choudhury N, Yoneda T, Choudhury
GG, Ghosh-Choudhury N (2011) Simvastatin prevents
skeletal metastasis of breast cancer by an antagonistic
interplay between p53 and CD44. J Biol Chem
286: 11314-11327.

Michishita M, Akiyoshi R, Yoshimura H, Katsumoto T,
Ichikawa H, Ohkusu-Tsukada K, Nakagawa T, Sasaki N,
from canine mammary gland adenocarcinoma cell lines.

paradigm: implications in veterinary oncology. Vet J
205: 154-160.
Simvastatin modulates β-catenin/MDR1 expression...


