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Original article

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

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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).

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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 Argyle 2015), which are characteristics of high histological grade in both canine and human mammary carcinomas (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, Klopfleisch 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 HMG-CoA-reductase (Mandal et al. 2011). In fact, we ourselves have reported that simvastatin, a lipophilic statin, induces antiproliferative and chemosensitizing effects on spheres isolated from CF41.Mg canine mammary carcinoma cells which were related to an increase in p53 activity and a transient decrease in β-catenin expression (Torres et al. 2015). Moreover, there is evidence that simvastatin plus γ-tocotrienol induce a cytotoxic effect on drug resistant human breast cancer cells, which may improve the antitumour treatment response (Gopalan et al. 2013).

Since chemosensitizing effects exhibited by simvastatin, it was relevant for us to explore in more detail the role of β-catenin and MDR1 in response to this statin. The aim of this study was to assess the expression and phosphorylation of β-catenin and, MDR1 expression in spheres derived from CF41.Mg cells exposed to simvastatin.

Materials and Methods

Cell culture. CF41.Mg canine mammary carcinoma cells (CRL-6232; ATCC®, Manassas, VA, USA) were grown in DMEM high glucose (4.5 g/L) in presence of 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate. Spheres derived from CF41. Mg cells were cultured in ultralow attachment plates with culture medium serum-free DMEM/F12 containing 10 ng/ml bFGF, 10 ng/ml EGF, 5 µg/ml insulin, 4 µg/ml heparin, B27 and, 20 µg/ml penicillin, 20 µg/ml streptomycin and 0.05 µg/ml amphotericin B. All cultures were maintained in a humidified atmosphere with 5% CO_2 at 37ºC.

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 Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 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

Table 1. Primary antibodies used for western blot analysis.

Specificity	Clone	Source	Immunoglobulin subclass	Dilution
β-catenin[a]	14/β-catenin	Mouse	IgG_1	1:1000
Phospho-β-catenin[b]	–	Rabbit	–	1:1000
P-glycoprotein[c]	C219	Mouse	IgG_1	1:200
β-actin[d]	mAbcam 8226	Mouse	IgG_1	1:1000

[a] BD Biosciences Cat. 610154; [b] Cell Signaling Cat. 9561S; [c] Millipore Cat. 517310; [d] Abcam Cat. ab8226.

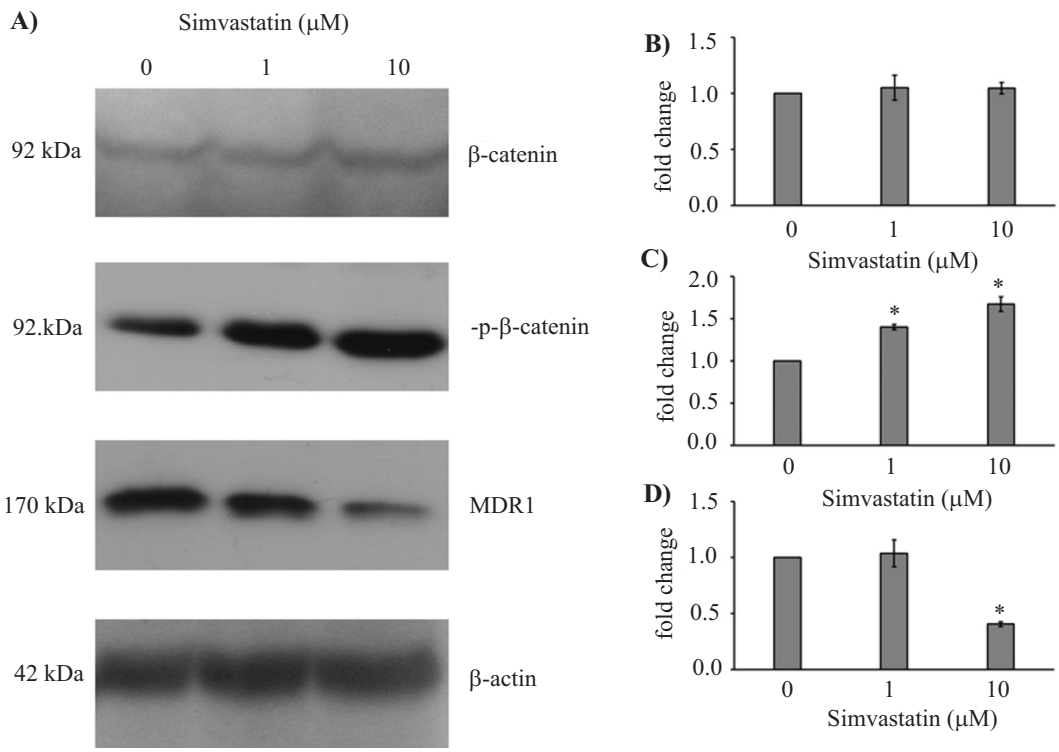

Fig. 1. Analyses of total and phosphorylated β-catenin and MDR1 proteins in spheres derived from CF41.Mg cells exposed to simvastatin. (A) Expression and phosphorylation of β-catenin and MDR1 expression in presence of 0-10 μM simvastatin for 48 h. (B-D) Histograms representing densitometric analyses of relative expression (protein levels normalized to β-actin) of β-catenin, p-β-catenin and MDR1 respectively. Representative results from three independent assays of cell extracts analysed by western blot. * Statistical significance at $p<0.05$ in relation to negative control.

room temperature and revealed with enhanced chemiluminescence (Renaissance Chemiluminiscence kit, Perkin Elmer). As a loading control, β-actin was detected. The immunoblot bands were analyzed with NIH Image J software version 1.41.

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 response 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 described (Torres et al. 2015). In that study, 10 μM simvastatin sensitized CSC to the cytotoxic effect of 250 ng/ml doxorubicin, a MDR1 substrate, causing more cell death than chemotherapeutic drug alone. This effect was observed at 72 h, an incubation time at which simvastatin did not induce cytotoxicity alone.

Simvastatin induced an increase in the phosphorylation of β-catenin, an effect that promotes its degradation and prevents its movement to the nucleus. The antibody used here identifies phosphorylated 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 downregulates MDR1 gene transcription with a consequent decrease in p-glycoprotein levels and the minor activity of this drug efflux pump. The effect of statins on MDR1 expression has been previously described by Sieczkowski's group, who verified that simvastatin decreased 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 observed 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 extracellular matrix (Chanmee et al. 2016). In addition, CD44 triggers activation of β-catenin, MDR1 and Bcl-xL expression, inducing chemoresistance (Bourguignon et al. 2009). Since there is evidence that simvastatin inhibits CD44 expression in human metastatic breast cancer cells (Mandal et al. 2011), it would be very interesting to explore the potential effects of simvastatin 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 therapeutic option against high grade canine mammary tumours and with potential application in human breast cancer; nevertheless, it is necessary to extend these results to other mammary carcinoma cells and carry out *in vivo* studies evaluating the findings described here.

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