The Veterinary Journal 205 (2015) 288–296

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Phenotypic screening of a library of compounds against metastatic and non-metastatic clones of a canine mammary gland tumour cell line

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ARTICLE INFO

Article history:
Accepted 19 April 2015

Keywords:
Canine
Mammary gland tumour cell lines
Metastases
Mitochondrial respiratory chain complex inhibitors
Chemotherapy
Compound screening

ABSTRACT

Metastases are associated with a poor prognosis for canine mammary gland tumours (CMGTs). Metastatic and non-metastatic clones were isolated previously from a single malignant CMGT cell line. The difference in metastatic potential between the two cell lines was hypothesised to be associated with distinct cellular signalling. The aim of this study was to screen for compounds that specifically target metastatic cells in order to improve CMGT therapeutic outcomes. The two clonal cell lines were characterised by transcriptome analysis and their sensitivity to a library of 291 different compounds was compared.

The metastatic clone exhibited elevated expression of molecules associated with degradation of the extracellular matrix, epithelial–mesenchymal transition and cancer stem cell phenotype. This was confirmed using a matrigel invasion assay and by assessment of aldehyde dehydrogenase activity. The mitochondrial respiratory chain complex inhibitors (MRCIs; rotenone, antimycin and oligomycin) significantly inhibited the growth of the metastatic clone. Although MRCIs similarly depleted mitochondrial ATP in both clones, the subsequent cellular response was different, with toxicity to the metastatic clone being independent of AMP-activated protein kinase activity. The results of this study suggest a potential utility of MRCIs as anti-tumour agents against metastatic CMGTs. Further studies are needed to investigate the clinical utility of MRCIs and to determine the association between MRCI sensitivity and malignancy.

Introduction

Metastasis is a critical prognostic factor in most forms of canine malignancy, including canine mammary gland tumours (CMGTs) (Chang et al., 2005). CMGTs are the most common neoplasm in sexually intact female dogs and account for approximately half of all tumours that develop naturally in these animals (Dorn et al., 1968; Rostami et al., 1994; Withrow et al., 2012). In dogs with CMGTs and macrometastasis in regional lymph nodes, overall survival (OS) after regional mastectomy is significantly shorter than that of dogs without metastases (Szczubiał and Łopuszynski, 2011). In a series of 72 post-mortem examinations of dogs with CMGTs, death was associated with the systemic spread of metastases rather than with local recurrence or invasion (Clemente et al., 2010). Therefore, prevention and control of metastasis is important for improving the prognosis of dogs with CMGTs.

Several studies using human and mouse tumour cells have suggested that not all tumour cells have the potential to migrate, invade, circulate and colonise to form metastatic foci (Garraway and Lander, 2013; Vogelstein et al., 2013; Kreso and Dick, 2014). A seemingly uniform tumour tissue contains numerous genetically and epigenetically heterogeneous cells. A clonal selection study using a mouse melanoma cell line showed that the metastatic potential of each tumour clone was strikingly different and that only certain clones have the ability to metastasise (Fidler and Kripke, 1977). This clonal heterogeneity and difference in metastatic potential has been shown with single cell resolution in a human colorectal carcinoma xenograft model (Dieter et al., 2011). Genetic analysis of clinical specimens also indicated intra- and inter-tumoural clonal diversity between primary and metastatic human malignancies (Gerlinger et al., 2012).

In a previous study by our group, two clonal cell lines with high or low metastatic potential were subcloned simultaneously from

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http://dx.doi.org/10.1016/j.tvjl.2015.04.025
1090-0233/

one parental canine mammary carcinoma cell line (Murai et al., 2012). This indicated that CMGTs also have clonal diversity and that only certain clones are responsible for metastases (Murai et al., 2012). Thus, metastatic clones in tumours might be an effective therapeutic cellular target and chemotherapy selective for these clones may improve treatment outcomes of CMGT.

Unbiased screening of chemical compounds is an approach used for exploring treatments that are selective against specific cell types (Gupta et al., 2009; Boichuk et al., 2014; Kitambi et al., 2014). This approach is based on the hypothesis that phenotypically different cell populations, such as cancer cells and their normal counterparts, have different cellular signalling and thus may have a different response to some compounds. We hypothesised that a metastatic CMGT clone has a distinct phenotype and unique cellular signalling when compared to a clone with lower malignancy. This, in turn, may result in differential sensitivity to particular compounds.

The aim of this study was to screen for compounds with differential activity against clones with different metastatic potentials in order to identify compounds that may improve chemotherapy for CMGTs. We first performed extensive expression analysis and phenotyping of two previously established CMGT clonal cell lines. Next, an unbiased compound screen was undertaken to select compounds that differentially affected the growth of the metastatic and non-metastatic clones. Finally, we explored the mechanism of action of the candidate compounds to gain insight into the specific pathways responsible for the observed differential effects.

Materials and methods

Cell culture

Metastatic CHMp-5b and non-metastatic CHMp-13a are clonal cell lines previously established using a limiting dilution method from the canine mammary carcinoma cell line CHMp (Murai et al., 2012). The cells were maintained in RPMI 1640 medium (Wako Pure Chemical Industries) supplemented with 10% foetal bovine serum (Life Technologies) and 50 μg/mL gentamicin (Sigma-Aldrich), and incubated at 37 °C in a humidified atmosphere with 5% CO_2.

cDNA microarray and real-time quantitative reverse transcription PCR

For expression analysis, total RNA was extracted from both cell lines during the exponential growth phase. Microarray analysis was performed as described by Tomiyasu et al. (2013) using a Canine ver. 2 (4 × 44K) array (Agilent Technologies). Several differentially expressed genes (DEGs) were also evaluated by quantitative reverse transcription (RT)-PCR (qPCR) to confirm the microarray data (Appendix: Supplementary Table S1). These genes were selected based on their reported association with tumour malignancy, prognosis or cell differentiation in epithelial malignancies. Full procedures and analytical details are provided in the supplementary material (see Appendix: Supplementary material).

Matrigel invasion assay

The Boyden chamber assay was adopted to compare the invasive capacity of the cells. Culture inserts (24-well permeable support, 8.0 μm pore, Corning) were set on a 24-well companion plate (Corning) to separate the well into upper and lower chambers. Matrigel (200 μL; 500 μg/mL; Becton Dickinson), which mimics the extracellular matrix, was added to the upper chamber and incubated for 2 h at 37 °C. Wells without the matrigel layer were used to count overall migrating cells through the pores. After removal of the unsolidified residual matrigel mixture, a cell suspension containing 2.5×10^4 cells in 500 μL serum-free RPMI medium was added to the upper chamber. RPMI medium supplemented with 10% foetal bovine serum was added to the lower chamber as a chemoattractant. The plates were incubated for 20 h at 37 °C in a humidified 5% CO_2 atmosphere to allow cells to invade into the matrigel and migrate to the lower chamber through the pores.

The culture inserts were fixed and stained with phosphate buffered saline (PBS) containing 6% glutaraldehyde and 0.5% crystal violet. After fixation, the cells in the upper chamber (non-migrating or non-invading cells) were removed gently using a cotton swab. Then, the centre of the membranes (lower chamber) was captured under a light microscope at 400× magnification, and the number of cells was counted. Data from four different membranes for each condition were collected, and the number of matrigel invading cells was divided by the averaged number of migrating cells.

Measurement of aldehyde dehydrogenase activity

Aldehyde dehydrogenase (ALDH) activity was measured using an Aldefluor assay kit (StemCell Technologies) as previously described (Michishita et al., 2012) to evaluate cancer stem cell phenotype. Cells were suspended at a final density of 1×10^6 cells/mL in Aldefluor assay buffer containing 1 μM ALDH substrate (boron dipyrromethene aminoacetaldehyde), with or without the ALDH inhibitor diethylaminobenzaldehyde (DEAB), and incubated for 40 min at 37 °C. ALDH activity (green fluorescence intensity) was determined by flow cytometry using FACSAria and FACSDiva software (Becton Dickinson). The threshold was established according to the experiment with DEAB. The percentage of cells with higher ALDH activity than the thresholds in the absence of DEAB (the $ALDH^{high}$ cells) was recorded.

Inhibitor screening, cell viability assay and colony forming assay

To find metastatic cell selective agents, unbiased inhibitor screening was performed using kits obtained from the Screening Committee of Anticancer Drugs (SCADS) (kit 1, ver. 2.3; kit 2, ver. 1.3; kit 3, ver. 1.2; kit 4, ver. 1.0). The kits include a total of 321 drugs and are composed of 291 unique drugs when the overlap drugs between the different kits are omitted. Cells ($n = 2000$) were seeded in each well of a flat-bottom 96-well plate. After incubation for 24 h, drugs were added under the following conditions; kits 1–3 at 0.1 μM, kit 4 at 0.01 μM (low concentration) or kit 1–4 at 1.0 μM (high concentration). Since kit 4 is composed of the panel of the approved small molecule inhibitors, this kit was used at lower concentrations. After 48 h incubation, cell viability was analysed using Cell Counting Kit-8 (Dojindo Laboratories). Absorbance was measured using a plate reader (ARVO MX, PerkinElmer) (Appendix: Supplementary material).

Quantification of ATP from oxidative phosphorylation

Cellular ATP content was measured to evaluate the effect of the candidate agents on cellular aerobic ATP production. Cells were suspended at 200,000 cells/mL in glucose-free RPMI (Sigma-Aldrich) supplemented with 2 g/L galactose (Sigma-Aldrich) to prevent ATP production from anaerobic glycolysis. A 100 μL aliquot of the cell suspension was added to a Nunc Luminunc 96-well plate (Thermo Fisher Scientific) and incubated for 24 h. Drugs were added and the amount of ATP in each well was quantified after 2 h by Mitochondrial ToxGlo Assay according to the manufacturer's instruction (Promega Corporation). Luminescence was measured using a plate reader (ARVO MX, PerkinElmer).

Analysis of reactive oxygen species

Production of reactive oxygen species (ROS) was monitored to evaluate the disruptive effect of the candidate agents on mitochondrial oxidative phosphorylation. ROS production was measured using 2′,7′-dichlorofluorescein diacetate (10 μM; Wako Pure Chemical Industries) after 30 and 60 min drug exposure. For longer drug exposures, ROS production was assessed with the Total ROS/Superoxide Detection Kit (Enzo Life Sciences) (Appendix: Supplementary material).

Western blot analysis

Western blot analysis was used to evaluate activation of AMP-activated protein kinase (AMPK) following exposure to the compounds. Control and treated CMGT cells were lysed and 10 μg of protein was resolved by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Appendix: Supplementary material).

Statistical analysis

All data are shown as means ± standard deviation (SD). The statistical methods and software used were two-sided Student's *t* test performed using Excel 2013 (Microsoft) and Dunnett's test performed using R software (R Development Core Team, 2013) and multcomp R package (Hothorn et al., 2008). In addition, Fisher's exact test was performed using R software to evaluate the enrichment significance of the results from the inhibitor screening. The choice of the statistical methods is described in each figure legend. $P < 0.05$ was considered statistically significant.

Results

Expression profiling and phenotype characterisation

The two clonal cell lines used in this study had clearly distinct transcriptomes, with 2038 DEGs from 16,206 differently annotated genes (Figs. 1A, B). The DEGs with the greatest fold change in CHMp-5b cells revealed higher expression of genes associated with tumour cell mobility, invasive capacity, epithelial–mesenchymal transition (EMT) or drug efflux, such as *MMP1*, *PLS3*, *BMP2*, *MMP3*,