NEOPLASTIC DISEASE

Platelets Inhibit Migration of Canine Osteosarcoma Cells

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Summary

The interaction between platelets and tumour cells is important for tumour growth and metastasis. Thrombocytopenia or antiplatelet treatment negatively impact on cancer metastasis, demonstrating potentially important roles for platelets in tumour progression. To our knowledge, there is no information regarding the role of platelets in cancer progression in dogs. This study was designed to test whether canine platelets affected the migratory behaviour of three canine osteosarcoma cell lines and to give insights of molecular mechanisms. Intact platelets, platelet lysate and platelet releasate inhibited the migration of canine osteosarcoma cell lines. Addition of blood leucocytes to the platelet samples did not alter the inhibitory effect on migration. Platelet treatment also significantly downregulated the transcriptional levels of *SNAI2* and *TWIST1* genes. The interaction between canine platelets or molecules released during platelet activation and these tumour cell lines inhibits their migration, which suggests that canine platelets might antagonize metastasis of canine osteosarcoma. This effect is probably due to, at least in part, downregulation of genes related to epithelial−mesenchymal transition.

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Introduction

The interaction between platelets and tumour cells is important for tumour survival and metastasis. Studies using human and murine tumour cell lines have shown that thrombocytopenia, antiplatelet treatment or dysfunctional platelet adhesive proteins reduced pulmonary metastatic burden in mice (Gasic *et al.*, 1968, 1973; Pearlstein *et al.*, 1984; Jain *et al.*, 2007), demonstrating important roles for platelets in tumour progression. Recent studies have demonstrated that the direct signalling between platelets and epithelial cancers (i.e. carcinomas and adenocarcinomas) facilitates the metastasis of such tumours (Labelle *et al.*, 2011, 2014; Schumacher *et al.*, 2013). Nearly all of the studies demonstrating that platelets facilitate metastasis were performed using cancers of epithelial origin. Although the prometastatic effect of platelets on these tumours is evident, there is a lack of reliable information regarding the effect of platelets on the metastatic potential of mesenchymal tumours such as osteosarcoma.

Several spontaneously occurring canine tumours exhibit behaviour very similar to comparable human cancers. For this reason dogs have been proposed as appropriate models for studying cancer biology, assessing therapeutic targets and evaluating the diagnostic value of platelet-associated biomarkers for predicting and following cancer progression. As dogs routinely share the same environment as man and have a naturally shorter life span, the use of this species to study cancer progression holds several

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advantages. In addition to similar biological behaviour and response to treatment, cancer is a leading cause of death in owned dogs in the USA and there is a shared demand for the development of new diagnostic techniques and therapies, making clinical trials in dogs with spontaneously arising tumours a useful addition to the therapy development pipeline (Rowell *et al.*, 2011).

A prime example of the similarities between those two species is the occurrence of osteosarcoma. Despite its relatively low incidence in man (Howlader *et al.*, 2014), osteosarcoma is the most common primary malignant bone tumour affecting young and elderly patients (Mirabello *et al.*, 2009). It is an aggressive and highly metastatic cancer, with significant morbidity and a poor prognosis for long-term survival (Mirabello *et al.*, 2009). In dogs, osteosarcoma is the most common type of non-haemopoietic primary malignant bone tumour and has a highly metastatic behaviour (Guo *et al.*, 2007). Even after appropriate surgical and chemotherapeutic protocols only approximately 50% of affected dogs are still alive within the first year (Kirpensteijn *et al.*, 2002) and fewer than 20% of dogs survive for more than 2 years following diagnosis (Selvarajah and Kirpensteijn, 2010).

In order to fill the knowledge gaps discussed above and to further develop the dog as a cancer model, the aim of this study was to test whether canine platelets or their contents affect the migratory behaviour of three canine osteosarcoma cell lines.

Materials and Methods

Experimental Design

The present experiment used three canine osteosarcoma cell lines (OSCA-8, OSCA-40 and OSCA-78) established from dogs with spontaneously occurring osteosarcoma (Scott *et al.*, 2011). Platelets and leucocytes used for the cell treatments were isolated from the whole blood of healthy, 8-month-old, female Treeing Walker coonhound dogs. The four different treatments in the initial experiments were with intact purified platelets, intact platelets plus leucocytes, platelet lysate and platelet releasate. Treatments consisted of either 1×10^7 intact platelets or equivalent amounts of platelet releasate, lysate or controls added to 1×10^5 tumour cells. For the platelet plus leucocyte treatments, 1×10^6 blood leucocytes were combined with purified intact platelets from the same animal and then added to the tumour cells.

The effect of the different treatments on the migratory behaviour of the osteosarcoma cell lines and the gene expression of epithelial—mesenchymal transition (EMT)-related proteins was measured using migration assays and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), respectively. Sample size was calculated by power analysis using effect size ≥ 1.5 (these effect sizes were observed either in pilot or previous studies performed in our laboratory), type I error probability of 0.05 and desired power of 0.8.

Blood Collection

Venipunctures were performed from the jugular vein using a 20 gauge needle with blood taken either into a syringe containing 3.2% sodium citrate or a Vacutainer™ tube containing 5% EDTA for platelet and leucocyte preparations, respectively. Blood draws and sample handling were performed with extreme care in order to avoid in-vitro platelet activation.

Platelet and Leucocyte Sample Preparation

Purified platelet isolation was performed using the protocol described by Trichler *et al.* (2013) with modifications. This protocol results in highly purified platelets and when performed correctly does not cause platelet activation. Briefly, 5 ml of blood was centrifuged at 1,200 g for 2 min with minimum brake applied to obtain platelet-rich plasma (PRP). Next, approximately three-quarters of the upper layer of the PRP was collected and subjected to density gradient centrifugation for further purification and removal of leucocytes. For this, 3 ml of PRP were layered on top of a 1.063 g/ml iodixanol density barrier (OptiPrep™, Sigma—Aldrich, St. Louis, Missouri, USA) and then centrifuged at 350 g for 15 min at 20°C with no brake. One millilitre of the interface cloudy layer, which consisted of the purified platelet sample, was aspirated. A haemocytometer was used to count platelets manually and examine the sample for leucocyte and erythrocyte contamination.

To prepare platelet fractions, intact platelets were either activated using 1 U/ml thrombin (Chronolog Corporation, Havertown, Pennsylvania, USA) for 10 min at room temperature, or lysed by ultrasonication with 10 bursts of 5 sec each at intervals of 60 sec and at 50% amplitude (Q125 Sonicator, QSonica LLC, Newtown, Connecticut, USA). The pellet fractions from the releasate and lysate were both separated from their supernatants by centrifugation at 10,000 g for 10 min at 4°C. Leucocyte isolation was performed using a density centrifugation protocol (Strasser *et al.*, 1998). Purified blood leucocytes were counted and examined for platelet and erythrocyte contamination with a haemocytometer. Leucocyte