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Research Paper

Isolation of circulating tumor cells in a preclinical model of osteosarcoma: Effect of chemotherapy

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ABSTRACT

Osteosarcoma is a rare primary bone tumor, which mainly affects children and adolescents and has a poor prognosis, especially for patients with metastatic disease. A poor therapeutic response to the conventional chemotherapy is observed with the development of lung metastases, highlighting the need for improving the current regimens and the identification of early markers of the recurrent and metastatic disease. Circulating Tumour Cells (CTCs) play a key role in the metastatic process and could be powerful biomarkers of the progressive disease. The present study aimed to isolate CTCs by using a pre-clinical model of human osteosarcoma and to monitor their kinetic of release and their modulation by ifosfamide. CTCs were detectable into the bloodstream before any palpable primary tumors. Ifosfamide increased CTCs count and in contrast decreased the number of lung tumor nodules. On established tumors, ifosfamide slowed down the tumour growth and did not modulate CTC count that could be explained by a release of cancer cells from the primary tumour with reduced properties for inducing lung metastases. This report highlights the biological interest of CTCs in osteosarcoma.

1. Introduction

Osteosarcoma is the most common primary malignant tumour of bone in children and young adults. This neoplasm has its origin in mesenchymal cells committed to the osteoblast differentiation programme [1–3]. Malignant cells are able to destabilize the accurate osteoblastic/osteoclastic activity balance of bone formation and resorption that in turn facilitates the tumour cell proliferation and formation of tumoral osteoid tissue. Conventional therapy for osteosarcoma patients combines neoadjuvant chemotherapy followed by surgical removal of all detectable disease (including metastases), and post-operative chemotherapy [1,4,5]. Current regimens of chemotherapy are based on four drugs: i) high-dose methotrexate (HDMTX) with leucovorin rescue; ii) doxorubicin (adriamycin); iii) cisplatin, and iv) ifosfamide [15]. Unfortunately, a low response rate to chemotherapy is frequently observed leading to the development of metastases preferentially located in the lungs and finally to the patient death.

The cell dissemination process is a very complex mechanism that involves various components of the tumour microenvironment (e.g. blood vessels, immune cells) [7–9]. Cancer cells that have escaped from the primary tumour and are able to invade the bloodstream to become Circulating Tumour Cells (CTCs), that will be capable to intravasate, survive in the circulation, migrate into the interstitial space and finally establish the tumour growth at a new location [10–12]. Only a small number of cells will successfully complete all the steps, thus it is important to identify and well characterize which CTCs are able to complete the metastatic process [12].

Numerous studies have underlined the biological value of CTCs that can be used as biomarkers in the follow up of epithelial malignancies such breast and prostate cancers [13]. CTCs, which are collected, isolated and characterized from a non-invasive liquid biopsy, reflect the primary or/and metastatic disease for which a targeted therapy may be given [13,14] Even if membrane vimentin or bone isoenzyme of alkaline phosphatase appear to be expressed by a maojority of bone sarcomas and could be used for isolating sarcoma CTCs [15–17], there is no universal marker for the CTC detection in tumours of mesenchymal

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origin. Consequently, various methods independent of any cell markers have been proposed and are based on size or density discrimination [18–20]. In light of the few reports available in the literature [15–17] and the lack of specific marker for isolating sarcoma CTCs, by using a preclinical model of osteosarcoma, the aims of the present study were to: i) isolate CTCs; ii) enumerate CTCs during the tumour growth and iii) determine the effect of ifosfamide treatment on CTC release.

2. Material and methods

2.1. Cell culture

HOS-MNNG human osteosarcoma cell line purchased from the American Type Culture Collection was modified for overexpressing the green fluorescence protein (GFP) [21]. GFP-MNNG/HOS cells were cultured in DMEM (Lonza, Belgium) supplemented with 10% FBS in a humidified 5% CO_2/air atmosphere at 37 °C.

2.2. In vivo experiments

For both experiments 6-week-old female athymic nude mice were purchased from Elevages Janvier (France) and Harlan Laboratory (UK) and procedures involving animal handling and care were approved by the Animal Care and Ethics Committee of the French Ethical Committee (CEEA.PdL.06, authorization number: 1280.01) and the Home office in UK [PPL: 70/8967, Establishment license no.: 50/2509]. Mice were acclimatized for at least one week prior to experimental manipulation. Mice were anesthetized by inhalation of Isoflurane/air (1.5%, 1 $Lmin^{-1}$) before i.m. inoculation of 1.5×10^6 GFP-MNNG/HOS cells resuspended with phosphate-buffered saline (PBS). Osteosarcoma cells were injected in close proximity to the tibia, rapidly leading to tumour growth in the soft tissue with secondary contiguous bone invasion and development of lung metastases [22]. The tumor volumes were monitored twice a week and calculated by measuring two perpendicular diameters using calipers, according to the following formula: $V = 0.523 \times L \times (S)^2$, in which L and S are, respectively, the largest and the smallest perpendicular diameters. Tumor volumes were monitored until a maximum of 2500 mm^3. Blood samples were collected in EDTA tubes by intracardiac puncture in anesthetised animals and mice were euthanized by cervical dislocation. Ifosfamide (Holoxan, Baxter) was prepared extemporaneously, diluting with PBS and administered intraperitoneally at doses of 15 mg/kg or 30 mg/kg per day on cycles of 3 consecutive days. Control mice were injected in the same conditions with an equivalent volume of PBS. The doses used were defined on the most commonly-used dose in the literature [23–26], on the frequency applicable in clinical practice, and on the recommendations proposed by Reagan–Shaw et al. [27]. Lung metastastatic nodules were macroscopically and manually scored in each animal at the time of necropsy as previously shown [22].

2.3. Blood processing: enrichment step of blood samples and isolation of circulating tumour cells

Collection of CTCs from blood combined a pre-enrichment step and an isolation phase. Pre-enrichment was carried out as follow: i) red cell depletion carried out by incubation of blood samples with cell lysis buffer for 10 min at room temperature; ii) cell wash with PBS three times consecutively; iii) leukocytes depletion using double positive selection with CD45- and Tert19-microbeads (Milteny Biotec, Germany) following the manufacturer's protocol. CTCs were the isolated either by fluorescence activated cell sorting (FACS ARIAIII, Becton Dickinson) or by the DEPArray™ platform (Menarini, Silicon Biosystems, Italy) to obtain pure single cells [28] (Fig. 1).

2.4. RNA extraction and gene expression analysis

Total RNA was extracted using the NucleoSpin® RNA II kit (Macherey-Nagel Gmbh & Co. Kg, Germany). RNA was reverse transcribed using the Maxima H Minus First Strand cDNA Synthesis kit (Thermo Scientific, France) following the manufacturer's instructions. Real-time PCR was performed with a CFX96 real-time PCR detector system (Bio-Rad, France) with SYBR Select master Mix reagents (Life Technologies, France). Primer sequences are detailed in supplementary data 1.

2.5. Proliferation assay

Proliferation was determined by using a WST-1 colorimetric test based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in viable cell and according the manufacturer's recommendation (Takara, Japan). Two thousands cells per well were seeded into 96 multi-well plates and after 24 h, 48 h and 72 h, cell proliferation was assessed by optical density measurement at 470 nm with Victor 2 1420 (Multilabel Counter, Perkin Elmer).

2.6. Migration and invasion assays

To evaluate migration ability of GFP-MNNG/HOS cells and isolated CTCs, cells were seeded in growth media without serum on the upper side of a Transwell chamber (Falcon) on a porous transparent polyethylene terephthalate membrane (8 μm pore size). The lower chamber of the transwell was filled with growth medium containing 10% FBS. After 24 hours of incubation, cells on the upper side of the filters were mechanically removed and cells migrated to the lower side were fixed (with glutaraldehyde 10%) and stained (with violet crystal 0.1%). Five random fields were taken under light microscope and analysed for each chamber and cells were counted with the ImageJ software (NIH, USA). The same procedure was used for invasion assays seeding cells on the upper side of Matrigel® (Corning, France)-coated transwells (50 ng Matrigel/well).

2.7. Statistical analysis

Data analysis was performed using GraphPad Prism (GraphPad Software, Inc). SEM from replicate experiments was calculated as noted in the legends and is shown as error bars. All error bars show SEM for at least triplicate measurement from independent experiments. The mean ± SEM was calculated for all groups and compared by non-parametric Mann–Withney Wilconson test. SPSS statistics software (IBM) was used for all statistical analysis. Differences were considered significant at the 95% confidence level ($p < 0.05$).

3. Results

3.1. CTCs are detectable before the tumour mass in a murine model of osteosarcoma

To validate the methods used and to avoid any bias linked to the mode of cancer cell injection in mice, 2×10^7 GFP-MNNG/HOS cells resuspended in PBS or Matrigel® were inoculated intramuscularly in paraosseous site. After blood collection, the presence of CTCs was analysed by flow cytometry after 24 h and 48 h post inoculation. In all conditions used, no CTCs were observed into the blood demonstrating that CTCs further detected were not associated to a technical artefact such as a leak of cancer cells into the bloodstream immediately after cell inoculation (data not shown).

We carried out a kinetic analysis of CTC release into the bloodstream. Thirty mice previously injected with 1.5×10^6 GFP-MNNG/HOS cells, were randomly divided into 3 groups and sacrificed at days 10, 30 and 45 respectively after cell injection (Fig. 2A). For each group,