Abstract. Aim: To identify a marker of osteosarcoma metastasis and to inhibit the marker against the invasive ability of an osteosarcoma cell line (143B). Materials and Methods: Type I insulin-like growth factor receptor (IGF-1R) and its downstream signalling factors were measured in samples from our orthotopic 143B mouse model by immunohistochemistry. A Matrigel assay was used to assess cell invasion ability under interference. Results: All 15 mice had tumour mass at the left tibia. Total IGF-1R, MEK, Akt, p38 and phosphorylated MEK (p-MEK), but not p-Akt and p-p38, were positive in both local tumours and lung secondaries. Leiomyosarcoma controls expressed p-Akt and p-MEK, but not p-p38. The 143B cells treated with U0126, a MEK/ERK inhibitor, had significantly lower in vitro invasion ability compared with controls. Conclusion: The IGF-1R-MEK signalling pathway, particularly Ras/Raf/MEK/ERK, may play an important role in osteosarcoma lung metastasis, and the targeting MEK/ERK by its specific inhibitor may have a potential use in the effective treatment of osteosarcoma.

Osteosarcoma is the most common primary malignant bone tumour. Despite combination therapy (multi-regime chemotherapy combined with surgery), about one-third of osteosarcoma patients with localised disease at initial diagnosis experience relapse (1), around 90% of these recurrences being due to lung metastases. The conventional therapy for non-metastatic osteosarcoma has achieved a 5-year survival of 60-70%, which has achieved little in two decades (2), but long-term survival for patients with metastatic or recurrent disease is <20% (3). Thus, there is a need for development of new treatment modalities.

Identifying key cellular signalling pathways associated with osteosarcoma progression and metastasis is important for the development of new targeted therapies. It has recently been found that the insulin-like growth factor (IGF) system plays an important role in the formation and homeostasis of bone (4). In osteosarcoma tissue samples and cell lines, differential expression of IGF-1 and IGF-2, and a more consistent expression of IGF-1R have been observed (5, 6). Targeting IGF-1R appeared to be effective in preclinical models, supporting that the IGF system may be useful target for cancer treatment (7, 8).

IGF signalling regulates cell survival, proliferation and metabolism. Interaction of ligand (IGF-1) and receptor (IGF-1R) causes phosphorylation of the tyrosine kinase domain of the receptor, leading to phosphorylation of intracellular proteins. The Ras/Raf/MEK/ERK, Ras/Raf/MEK/p38-MAPK, and Ras/PI3K/Akt/mTOR are major pathways contributing to tumourigenesis, maintenance of phenotype, and protection from apoptosis (9). Specific pathway signalling factor inhibitors (e.g. U0126 for MEK/ERK) are commercially available and have been used in studies of other types of cancer (10).

Since imbalanced regulation of the IGF system may play an important role in osteosarcoma, we hypothesized that some IGF signalling pathways may be associated with lung metastasis of osteosarcoma. This study aimed to (i) set up an orthotopic mouse model of human osteosarcoma with spontaneous pulmonary metastasis; (ii) identify metastatic associated pathway by detecting protein expression of IGF-1R and its downstream signalling factors: Akt, MEK1/2 and p38 MAP kinase (total and phosphorylated proteins), at the primary site (tibia) and a metastasis (lung), and (3) assess the effect of inhibitory treatment of the significant pathway marker on in vitro invasion by the osteosarcoma cell line used for the in vivo model.

Materials and Methods

Cell culture and animal housing. The human osteosarcoma cell line 143B was cultured in RPMI-1640 medium supplemented with 10% FBS and antibiotics. Fifteen 5-week-old female Balb/c nu/nu mice
Animal Resource Centre, Perth, Australia) were used. The study was approved by the University of New South Wales Animal Care and Ethics Committee and was performed in accordance with the National Health and Medical Research Council guidelines for the use of animals for scientific research.

Tumour cell inoculation and growth observation. Individual mice were anaesthetized with isoflurane (1-3%) and oxygen (100%) inhalation. A 0.5 cm longitudinal skin incision was made along the left tibia, from the distal end of the patella tendon, to expose the proximal tibia. The cortical layer of the bone was punched through.

Figure 1. Tumour growth curve is shown as the mean tumour volume plus standard deviation at different time points (a). The gross view of the tumour at the primary sites (b-d) and lung (e, f). Abundant blood supplies to local tumours at 4 (c) and 6 (d) weeks were noted. Multiple cysts were formed at 6 weeks (d). Multiple nodules were visualized in lungs at 4 weeks (e: front view, f: back view).

Figure 2. X-ray revealed bone resorption and deformation of the left legs at the local tumour sites, which progressed with tumour growth at 2, 4 and 6 weeks (a, b, c). Tartrate-resistant acid phosphatase staining revealed the osteoclast activity at the margin of resorbed bone adjacent to tumour at 2 (d), 4 (e) and 6 (f) weeks.

Figure 3. Messenger RNA expression of human ALU gene in the tumour cells located in mouse tibias (a, b) and the lungs (c-e). The samples were collected at 2 (a, c), 4 (b, d) and 6 (e) weeks.

Figure 4. Detection of IGF-1R protein expression in a 2-week primary tumour (left) and 4-week lung metastasis (right) of the mouse model.
using a 27.5 gauge needle 2 mm below the patella tendon and $3 \times 10^5$ 143B cells in 10 μl of PBS was injected into the tibia. The skin was closed using EPIGLU (Meyer-Haake GmbH Medical Innovations, Wehrheim, Germany). The mice were continuously monitored and their general health, body weight and tumour size were recorded twice a week. Five mice were sacrificed at 2, 4 and 6 weeks after inoculation.

Gross assessment and X-ray imaging. At sacrificing, the hind limbs and lungs were examined. The tumour sizes were measured with a pair of calipers. The tissues were harvested and plain X-ray was applied to the limbs using a radiophotograph system (MX20/DX50, Faxitron X-ray Corporation, Wheeling, IL, USA) at 17 kV/60 s.

Histology. The formalin-fixed hind limbs were decalcified and the tibias were halved sagitally, embedded in paraffin and sectioned at 5 μm. The sections were stained with Harris’ haematoxylin and eosin (H&E) for histological analysis. Osteoclast activity was identified by tartrate-resistant acid phosphatase (TRAP) staining. The lung specimens were embedded and sectioned. Every fifth section was H&E stained for tumour screening.

Immunohistochemistry (IHC) and in situ hybridization (ISH). Standard IHC was applied to the tumour sections. Polyclonal rabbit antibodies against human IGF-IRα (N-20) from Santa Cruz (CA, USA); MEK1/2, phosphor(p)-MEK1/2 (Ser217/221), Akt (pan) (C67E7), p-Akt (Ser473), p38 MAPK and p-p38 MAPK (Thr180/Tyr182) from Cell Signalling (Danvers, MA, USA) were used. Human leiomyosarcoma samples, which previously showed positive staining of these markers, were used as positive tissue controls and non-immunized rabbit serum was used as a non-specific Ig control. A horseradish peroxidase (HRP)-conjugated secondary anti-rabbit antibody (EnVision, Dako Cytomation, Sydney, Australia) and Dako liquid DAB* were followed by H&E counterstaining. For identifying the origin of tumour cells, the sections with xenografted tumours were tested for human ALU mRNA expression using a Universal ISH Kit (BioGenex, San Ramon, CA, USA) with HRP and DAB for visualization.

In vitro invasion assay and statistical analysis. 143B cells ($5 \times 10^3$ per well) were added in duplicate to the Matrigel-coated polycarbonate filter wells (6.5 mm wide/8 μM pore size), in the chamber of a 24-transwell plate (Corning Costar, Cambridge, MA, USA), in serum-free media with either MEK/ERK inhibitor (U0126 at 10 nM) or vehicle control (0.06% DMSO). The plate was incubated at 37°C for 72 hours. Cells that migrated to the lower surface of the membranes were then stained with 0.5% crystal violet in PBS. Positively stained cells from 10 random fields of the duplicate wells (5/well) were counted. Independent t-test with Bonferroni’s correction in the PASW Statistics 18 software (SPSS Inc., Chicago, IL, USA) was used for comparison of difference between U0126 and control groups. P-values (two-tailed) <0.05 were considered significant.
Results

Establishment of an orthotopic mouse model of osteosarcoma. Local tumour growth was noted at day 7 post inoculation. The tumour sizes increased in a geometric progression (Figure 1a). No signs of mouse depression were noted. Gross examination at mouse sacrificing showed that at 2 weeks, a hard nodule had formed from the proximal tibia, with sizes ranging from 2×2×3 mm to 12×6×5 mm (Figure 1b). At 4 weeks, the local tumour growth had reached 17×11×10 mm, on average, and they were localised within a membranous tissue capsule with an abundant blood supply (Figure 1c). At 6 weeks, a huge local tumour mass (23×21×20 mm on average) was observed (Figure 1d). The lumps were still encapsulated and multiple cysts were formed underneath the membrane felt by palpation. Multiple nodules in the lungs were noted from week 4 (Figure 1e and f) and after.

Characterization of the mouse model of osteosarcoma and lung metastasis. X-ray revealed bone resorption at the proximal tibia of the left legs in all 5 cases at 2 weeks (Figure 2a). No fracture or deformation of the bone was noted at this time point. At 4 weeks, the proximal tibias were resorbed, with the resorption extending to the knee joints and femurs, causing joint deformation and segmental bone defects (Figure 2b). At 6 weeks, there was hardly any bone left in the left hind limbs (Figure 2c). TRAP staining demonstrated osteoclast activity at the margin of resorbed bone adjacent to tumour at 2, 4 and 6 weeks (Figure 2c-e). Pulmonary metastasis was visualized in the mice at 4 and 6 weeks. No lung metastasis was found microscopically in any of the mice at two weeks. Four out of 5 mice (80%) at 4 weeks and all mice (100%) at 6 weeks showed microscopic lung metastasis. Human ALU expression confirmed the human origin of the tumour cells (Figure 3).

Protein expression and metastasis association. IHC revealed positive staining in the primary tumour and lung metastasis of total proteins of IGF-1R, MEK, Akt and p38 MAPK. Expression of IGF-1R is shown in Figure 4. Different from their total protein expression, only p-MEK tested positive in both primary tumour (Figure 5a and Figure 5b) and lung metastasis (Figure 5c and Figure 5d), whilst P-Akt (Figure 5e and Figure 5f) and p-p38 MAPK (Figure 5g) were negative. Human leiomyosarcoma control samples expressed p-Akt (Figure 5h) and p-MEK, but not p-p38. The experiments were repeated with a two-fold higher concentration of each primary antibody against the phosphorylated proteins and similar results were achieved. IGF-1R expression was mainly on the tumour cell membrane, whilst that of total proteins MEK, Akt and p38-MAPK, were mainly in the nucleus. The staining intensity increased with time. p-MEK was also found in the tumour cell nuclei.

Regulation of in vitro invasion. Because one of the MEK pathways, MEK/p38 MAPK, may not be involved, based on p-p38 MAPK staining being negative, we assessed whether inhibition of MEK/ERK pathway by its specific inhibitor (U0126) would interfere with the ability of the targeted 143B cells to invade Matrigel in vitro. The U0126-treated cells were found to be significantly less invasive than untreated control cells (Figure 6a versus Figure 6b; Table I).

Discussion

Osteosarcoma presents a therapeutic challenge in oncology due to its high rate of metastasis and patients’ intolerance to the toxicity from the current conventional therapies. New molecular targets need to be identified to develop effective therapies and improve patient outcomes in the future. Human osteosarcoma mouse models are preferably used by researchers nowadays to identify genetic and biomolecular factors that are closely associated with tumour progression and metastasis (11). The 143B cell line is one of most commonly used cell lines and the orthotopic inoculation of cells is the most preferred method, as the tumour cells are made to propagate from the bone and they eventually spread, mimicking a spontaneously metastasizing process in patients (11). Our study confirmed that the orthotopic 143B mouse model is a reliable model for studying osteosarcoma growth and metastasis, and investigated the associated biomolecules.

The 143B cell line was generated via a K-RAS oncogene transformation of the HOS osteosarcoma cell line (11). Major Ras downstream signalling includes the Ras/Raf/MEK/ERK,
Ras/Raf/MEK/p38-MAPK, and Ras/PI3K/Akt/mTOR pathways. Our study demonstrated that although total protein expression of IGF-1R, MEK, Akt and p38-MAPK was positive, only p-MEK, but not p-Akt and p-p38 tested positive in both primary and lung metastasis. Control tissue samples were positive for p-Akt, suggesting the antibody against p-Akt was working. No specific antibodies against activated IGF-1R were available. These results suggest that overactivation of the Ras/Raf/MEK/ERK pathway may be responsible for osteosarcoma lung metastasis in this model. Consistently, other researchers have indicated that the three Ras signalling pathways do indeed act differently. The MEK/p38 pathway promoted cancer cell differentiation (12). The Ras/PI3K/Akt pathway is required for protection from apoptosis (13). In contrast, the Ras/Raf/MEK/ERK pathway is required for epithelial–mesenchymal transition (EMT), tumourigenesis, and metastasis (14). How does this pathway promote tumour metastasis? It is known that the pathway plays an important role in many steps of cancer metastasis, such as expression of integrin receptors for extracellular matrix proteins (15), production of proteases (e.g., MMP-9), the EMT, and regulation of endothelial apoptosis/angiogenesis (16).

The Ras/Raf/MEK/ERK signalling may be a key factor not only for metastatic osteosarcoma but for general metastasis of cancer, since this pathway is present in all eukaryotic cells (17) and relays extracellular signals through IGF-1R receptor to the nucleus via Ras, Raf, MEK, and ERK. Additionally, by stimulating transcription factors such as AP1, which acts on more than 50 substrates in the cytosol and the nucleus, it regulates fundamental cellular processes including proliferation, differentiation, survival, angiogenesis, adhesion, mobility and transformation (18). Although few studies involved osteosarcoma, it is known that the Ras/Raf/MEK/ERK pathway is up-regulated in approximately 30% of other human cancer types and over 50% of all metastatic tumours caused by mutations in RAS or BRAF (19, 20). Cohort studies using breast carcinomas and head and neck squamous cell carcinomas reported significant correlations between the level of activated MEK/ERK expression and the presence of lymph-node metastases (21, 22).

Since the Ras/Raf/MEK/ERK pathway is important in promoting tumour metastasis, we hypothesised that inhibition of MEK/ERK should translate into a measurable reduction in osteosarcoma invasive/metastatic ability. Our results demonstrated that MEK/ERK inhibition by U0126 (15) significantly reduced the in vitro invasive ability of the targeted 143B cells more than untreated controls, suggesting this approach may have a potential implication in treatment of patients with osteosarcoma. To date, there has been no report on U0126 interfering with osteosarcoma invasion in vitro. However, studies in other types of cancer investigating the effect of U1026 inhibition on tumour invasion and metastasis in vitro support our results (10, 23, 24). Furthermore, this approach has been used in phase II clinical trials in colon cancer, in which there have been highly frequent upstream KRAS and BRAF mutations which continuously transduced activated signals to MEK/ERK and contributed to host cell malignant behaviour (25).

Detection of the human-specific ALU gene has been used to monitor disseminated human cancer cells in a background of large numbers of xenogenic host cells. Methods used in the literature all require extraction of genomic DNA, which include dot blot hybridization (26), PCR (27) and real-time PCR (28). Different from those methods, this study used an in situ hybridization assay to directly measure ALU mRNA expression in human cancer cells located in the mouse xenograft tissue samples. This is an easy and reliable method. Moreover, in this way, the distribution of human cancer cells in the involved mouse tissue can be seen straight away by microscopy.

In summary, we have successfully established a usable orthotopic mouse model of human osteosarcoma growth and metastasis. Using this model, we found that overexpression of the phosphorylated MEK, but not p-Akt and p-p38, was detected in the xenograft primary and metastatic tumours apart from the total IGF-1R and MEK proteins, suggesting that the Ras/Raf/MEK/ERK signalling is an important factor of osteosarcoma growth and metastasis. Furthermore, we used U0126 to inhibit MEK/ERK function, which resulted in significantly reduced in vitro invasive ability of the treated osteosarcoma cells. Our results may have a potential clinical implication in the prediction of metastasis and in the development of new targeted therapies in osteosarcoma.

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References


