Expression of 11β-hydroxysteroid dehydrogenase enzymes in human osteosarcoma: potential role in pathogenesis and as targets for treatments

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Abstract

Osteosarcoma (OS) is a primary malignant tumour of bone occurring predominantly in children and young adults. Despite chemotherapy, relapse is common and mortality remains high. Non-transformed osteoblasts are highly sensitive to glucocorticoids, which reduce proliferation and induce apoptosis. Previously, we observed that OS cells, but not normal osteoblasts, express 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). This enzyme inactivates cortisol (active) to cortisone (inactive) and expression of 11β-HSD2 renders OS cells resistant to glucocorticoids. By contrast, the related enzyme 11β-HSD1 converts cortisone to cortisol and reduces OS cell proliferation in vitro. Some synthetic glucocorticoids (e.g. dehydrodexamethasone (DHD), inactive counterpart of dexamethasone (DEX)) have been reported to be activated by 11β-HSD2. We therefore investigated expression and enzymatic activity of 11β-HSD isozymes in human OS tissue, determined whether 11β-HSD expression has prognostic value in the response to therapy, and evaluated the potential use of synthetic glucocorticoids to selectively target OS cells. OS samples expressed both 11β-HSD1 and 11β-HSD2. 11β-HSD1 expression in pretreatment biopsy specimens positively correlated with primary tumour size. Expression and activity of 11β-HSD1 in post-treatment biopsies were unrelated to the degree of tumour necrosis following chemotherapy. However, high 11β-HSD2 expression in post-treatment biopsies correlated with a poor response to therapy. OS cells that expressed 11β-HSD2 inactivated endogenous glucocorticoids; but these cells were also able to generate DEX from DHD. These results suggest that OS treatment response is related to 11β-HSD2 enzyme expression. Furthermore, OS cells expressing this enzyme could be targeted by treatment with synthetic glucocorticoids that are selectively reactivated by the enzyme.

Endocrine-Related Cancer (2012) 19 589–598

Introduction

Osteosarcoma (OS) is the most common primary malignant tumour of bone occurring in children and adolescents (Wafa & Grimer 2006). Surgical resection alone is associated with a survival rate of ~11% due to the presence of systemic micrometastases at presentation. However, the introduction of systemic chemotherapy regimes followed by excision of the primary tumour has increased survival rates between 60 and 80% in patients without detected metastases at diagnosis (Raymond et al. 2002). Most OSs are high grade and there are few morphological markers relating to long-term survival. Patients with metastases at presentation have a very poor outlook. For patients
without clinically evident metastasis, the most important prognostic factor is the response of the primary tumour to chemotherapy. The finding of >90% post-chemotherapy necrosis in the primary tumour is associated with a good prognosis (Marina et al. 2004, Wafa & Grimer 2006). Conversely, <90% necrosis is associated with a high probability of systemic relapse. Weaker prognostic factors include the size of the primary tumour and the level of serum alkaline phosphatase at diagnosis (Marina et al. 2004, Bramer et al. 2005).

OSs arise from cells of osteoblast lineage. Normal osteoblasts are highly sensitive to glucocorticoids, which have anti-proliferative, pro-differentiative and pro-apoptotic actions on these cells (Cooper 2004, O’Brien et al. 2004). The detrimental effects of therapeutic glucocorticoids are frequently observed in clinical practice and are manifest through increased risk of fracture/osteoporosis and osteonecrosis. In vitro experiments indicate that OS cells and cell-lines retain sensitivity to glucocorticoids (Kudawara et al. 2001). This suggests that OS in vivo could be sensitive (or sensitised) to glucocorticoids. At a cellular level, glucocorticoids are metabolised by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes (Tomlinson et al. 2004, Cooper & Stewart 2009). These intracellular enzymes interconvert hormonally active glucocorticoids with their inactive forms. The 11β-HSD1 enzyme metabolises endogenous glucocorticoids, cortisone and cortisol, in a bidirectional manner, whereas 11β-HSD2 is a unidirectional inactivator of endogenous glucocorticoids. We have previously demonstrated that OS cell lines express the 11β-HSD2 enzyme (Bland et al. 1999, Eyre et al. 2001). This is in contrast to normal osteoblasts that express the 11β-HSD1 enzyme (Cooper et al. 2000, 2001, 2002). Expression of 11β-HSD2 is also seen in pituitary tumours and this expression is related to prognosis (Rabbitt et al. 2003). This raises the possibility that differences in glucocorticoid metabolising enzyme expression, and in particular 11β-HSD2, could be relevant to OS prognosis or response to treatment.

Although 11β-HSD2 activity is unidirectional for endogenous glucocorticoids, several studies have demonstrated that the substitution of a fluoride molecule at the 9α position of the glucocorticoid structure (as seen in dexamethasone (DEX)) dramatically affects the way the enzyme metabolises these steroids (Diederich et al. 2002). 9α-Fluorinated steroids are less effectively oxidised (inactivated) but in addition their inactive counterparts can be reduced (converted to their active form) by the 11β-HSD2 enzyme. As such, it has been shown that 11β-HSD2 can efficiently convert dehydrodexamethasone (DHD; the inactive oxidised counterpart of DEX) and DEX in a bidirectional manner.

The aims of this study are to investigate the expression and enzymatic activity of the 11β-HSD isozymes in human OS tissue before and after chemotherapy, to determine whether 11β-HSD expression has prognostic value in terms of response to therapy, and to evaluate the potential use of synthetic glucocorticoids in selectively targeting OS cells in patients who have responded poorly to chemotherapy.

Materials and methods

Patients

OS biopsy and post-treatment resection specimens were obtained from the Department of Musculoskeletal Pathology at the Royal Orthopaedic Hospital. All subjects gave informed consent and the study was approved by the Warwickshire Research Ethics Committee. Histological preparations of tissue samples were identical to those used as part of the departmental clinical service. Patient diagnoses were based on assessment by a consultant pathologist in collaboration with the Orthopaedic Oncology Multidisciplinary Team. Specifically, all biopsy samples and resection specimen were formalin-fixed and decalcified in 5% nitric acid. Sections were stained with haematoxylin and eosin and examined microscopically. Diagnosis was based on the presence of matrix (osteoid/cartilage) producing frankly malignant sarcomatous stroma. Tumours with prominent osteoid were labelled as osteoblastic variant of OS. Tumours with more than 30% cartilaginous component were labelled as chondroblastic OS. To assess tumour size and percentage necrosis, all resection specimens were opened with a bandsaw and a slice through the entire bone with the tumour was taken. The slice was photographed and mapped. Blocks were submitted for histological evaluation. The effect of chemotherapy was assessed by the proportion of viable and necrotic areas and recorded in terms of the percentage of tumour necrosis. Tumour volume was estimated by multiplying tumour linear dimensions to give a volume in cubic centimetre. Serum alkaline phosphatase levels were recorded at the time of diagnosis and subsequently as a measure of treatment response. Patients were treated with chemotherapy regimens containing methotrexate, doxorubicin and cisplatin chemotherapy with or without ifosfamide and etoposide.
Immunohistochemistry

Using the Labeled Streptavidin Biotin (LSAB) + System–HRP technique (Dako, Ely, UK), immunohistochemistry was performed on formalin-fixed paraffin-embedded human OS biopsy and post-chemotherapy OS specimens. Positive control sections included normal human liver for 11β-HSD1 and normal human kidney for 11β-HSD2. All tissue sections were prepared at the Department of Musculoskeletal Pathology (Royal Orthopaedic Hospital NHS Foundation Trust). Tissue sections were placed in a 60 °C heat block for 1 h before dewaxing in xylene and rehydration. The activity of endogenous peroxidase was blocked by 1% hydrogen peroxide for 10 min. Antigen retrieval was achieved using 1×EDTA/NaOH buffer (pH 8) overnight for 16 h. Sections were rinsed in warm water followed by cold water, then placed into a Sequenza immunostaining chamber and rinsed in tris-buffered saline (TBS) for 5 min. Endogenous biotin was blocked using a biotin-blocking buffer (Dako). Sections were incubated with primary antibody for 11β-HSD1 (at a 1:300 dilution) and 11β-HSD2 (1:300) (both from Binding Site, Birmingham, UK) in TBS for 1 h at room temperature. Sections were rinsed in TBS for 5 min. Immunodetection was enhanced using the LSAB + System–HRP technique, where sections were incubated for 30 min with a biotinylated link solution for 30 min, followed by TBS rinse for 5 min and another 30 min incubation with streptavidin peroxidase. Sections are incubated with 3,3-diaminobenzidine substrate–chromogen for 5 min, followed by counterstaining of sections with Mayer’s haematoxylin. Enzyme expression was assessed semiquantitatively in a blinded fashion on a scale of 0 to 4, where 0, indicated no expression; 1, low expression; 2, medium expression; 3, high expression and 4, very high expression.

Enzyme activity assay

Enzyme activity assays were performed as previously reported (Hardy et al. 2008). Fresh tissue was collected from post-chemotherapy OS surgical resection specimens. Tissue samples were cut into 3–5 mm thick pieces and washed twice with sterile PBS. Tissue samples were treated with 100 nM cortisol or 100 nM cortisol in 500 μl DMEM/Ham’s F12 (PAA Laboratories, Yeovil, UK) at 37 °C for 24 h along with the respective tritiated tracer for each steroid. Tissue was removed and weighed after 24 h, steroids were extracted from the media using dichloromethane and then separated by thin layer chromatography (TLC) using chloroform/ethanol (92:8). Steroid conversion was measured on the Bioscan 3000 image analyser (Lablogic, Sheffield, UK). Enzyme activity was expressed as picomole per gram of tissue per hour. Experiments were carried out in triplicate.

RNA extraction, RT and real-time PCR

RNA was extracted from OS tissue using the Tri-reagent extraction method (Sigma). RNA was further cleaned using a Qiagen RNAeasy kit. One microgram of total RNA was reverse transcribed using a RT kit (Applied Biosystems, Warrington, UK). 11βHSD1, 11βHSD2 and alkaline phosphatase mRNA expression levels were quantified by real-time PCR using the ABI 7500 sequence detection system (Perkin-Elmer Applied Biosystems, Warrington, UK). Primer and probes for each gene including the housekeeping gene for 18S rRNA were purchased from Assay-on-Demand (Applied Biosystems). Reactions were run on Micro-Amp Optical 96-Well plates, with 10 μl reactions containing Taqman PCR master mix (Applied Biosystems) as per the manufacturer’s instructions.

Metabolism of synthetic glucocorticoids by 11β-HSD enzymes

Tritiated DHD ([3H] DHD) was synthesised from tritiated DEX ([3H] DEX (PerkinElmer, Warrington, UK)) using an in house method based on the 11β-HSD2 activity of human placenta (Cooper et al. 2000). Fifty microlitres placental homogenate were incubated with 20 μl of [3H] DEX, 50 μl 0.1 M potassium phosphate buffer (pH 7.6) and 380 μl 10 μM NAD at 37 °C for 24 h. Steroids were separated by TLC as previously described. Using the Bioscan imager and unlabelled DHD and DEX standards (observed on the basis of their intrinsic autofluorescence using a u.v. imager), the [3H] DHD peak was identified and extracted from TLC plate silica and resuspended in ethanol.

Metabolism of the synthetic glucocorticoids DHD and DEX was investigated in stably transfected HEK293 cells overexpressing 11βHSD2 (293T2). Briefly, cells were cultured in DMEM-high glucose supplemented with 10% FBS, pen/strep and 0.5 mg/ml geneticin (G418). Cells were treated with 100 nM DHD or 100 nM DEX with the equivalent tritiated tracers for 6 h for the assessment of enzyme activity. Similar experiments were carried out in the MG-63 OS cell line, which has previously been shown to have high levels of 11β-HSD2 expression (Bland et al. 1999). All experiments were carried out in triplicate on three separate occasions.
**Statistical analysis**

Pearson’s product moment correlation coefficient was used to examine associations between continuous variables. Spearman’s rank correlation coefficient was used to examine associations between non-continuous variables (e.g. semiquantitative enzyme expression). A $P$ value of $<0.05$ was considered significant. All analyses were performed with SigmaStat Software (Systat Software, Inc., San Jose, CA, USA).

**Results**

**Expression of 11β-HSD enzymes in pretreatment OS tissue**

Expression of 11β-HSD1 and 11β-HSD2 was determined in pretreatment biopsy tissue by immunohistochemistry. Both enzymes were detectable but each to a variable degree (Fig. 1A). In post-chemotherapy samples, expression of both enzymes was also evident although again this was to varying degrees (Fig. 1B). Expression of 11β-HSD enzyme protein by immunohistochemistry assessed semiquantitatively in a panel of OS biopsy specimens did not appear to relate to the subtype of OS. 11β-HSD enzyme mRNA was also demonstrated in osteoblastomas (non-malignant bone tumours) but to a much lesser degree (Table 1).

The associations between pretreatment OS tissue enzyme expression, serum alkaline phosphatase activity and estimated primary tumour volume were examined. There was no association between 11β-HSD1 expression and alkaline phosphatase (Fig. 2A). There was a trend towards an association of 11β-HSD2 expression and serum alkaline phosphatase ($r=0.57$, $P=0.06$). 11β-HSD1 (but not 11β-HSD2) expression was strongly associated with estimated primary tumour volume ($r=0.88$, $P<0.001$).

**11β-HSD enzymatic activity in post-chemotherapy resection specimens**

Owing to the limited amount of tissue available from the pretreatment biopsy specimens, enzyme activity and mRNA expression analysis were primarily carried out on post-chemotherapy resection specimens. Reductase activity (an activity exclusively mediated by the 11β-HSD1 enzyme) did not correlate with the level of necrosis (Fig. 3). Dehydrogenase activity (which could be mediated by either 11β-HSD2 or 11β-HSD1) was, however, significantly associated with percentage of necrosis with low necrosis associated with high dehydrogenase activity. This association was also clearly evident when samples were analysed according to whether there was less than or more than 90% necrosis. To address the issue of the origin of the specific activities within the tissue, the relationship between 11β-HSD mRNA expression and expression of alkaline phosphatase was explored. There was no correlation between 11β-HSD1 alkaline phosphatase mRNA expression. By contrast, there was a significant correlation between 11β-HSD2 and alkaline phosphatase mRNA expression. This indicated that the reductase activity was unlikely to be due to the residual OS tissue, but the dehydrogenase activity was due to 11β-HSD2 expression within OS cells.

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**Figure 1** Immunohistochemical analysis of 11β-HSD isozyme expression in human osteosarcoma tissue. Expression was examined in biopsy and resection specimens (A) before and (B) after chemotherapy. For each of three representative osteosarcoma samples staining for haematoxylin and eosin (H&E), 11β-HSD1 and 11β-HSD2 expression is shown. Liver and kidney samples were used as positive controls for 11β-HSD1 and 11β-HSD2 expression respectively.

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Glucocorticoid metabolism in OS cells

The metabolism of endogenous and synthetic glucocorticoids was examined in the MG-63 OS cell-line and the HEK293 cell-line transfected with 11β-HSD2 (293T2). As expected, both cell-lines displayed exclusive dehydrogenase activity with the endogenous glucocorticoids, cortisol and cortisone (Fig. 4A and B). By contrast, the metabolism of DEX/DHD was bidirectional. In OS cells, the major enzymatic activity was reductase. In 293T2 cells, the metabolism of DEX/DHD was clearly bidirectional with the dehydrogenase activity favoured. The $K_m$ and $V_{max}$ values for the dehydrogenase and reductase activities were in the nanomolar and micromolar ranges respectively (Fig. 4C). These studies confirm that OS cells have the capacity to generate active glucocorticoids from inactive precursors using selected synthetic glucocorticoids.

Discussion

Treatment options for OS are currently limited and the outcome after disease relapse after initial chemotherapy remains extremely poor. This study found that OS cells express the glucocorticoid-modifying enzymes, 11β-HSDs. Expression of both 11β-HSD1 and 11β-HSD2 enzymes was observed. Expression of the glucocorticoid-activating enzyme 11β-HSD1 in pretreatment specimens was strongly correlated with primary tumour volume, with low expression associated with greater tumour volume. 11β-HSD1 expression in post-treatment specimens did not correlate with response to chemotherapy, whereas high levels of the glucocorticoid-inactivating enzyme 11β-HSD2 in post-chemotherapy OS samples were associated with a poor response to chemotherapy. We have previously demonstrated that expression of the 11β-HSD2 enzyme is associated with a pro-proliferative action on pituitary tumour growth (Rabbitt et al. 2003). This raises the possibility that 11β-HSD2 expression and the corresponding reduced level of glucocorticoid exposure within the cell are an important determinant of OS aggressiveness. OS cells transfected with 11β-HSD1 proliferate more slowly than cells with low 11β-HSD1 expression (Rabbitt et al. 2002). This could account for the observation

<table>
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<tr>
<th>Diagnosis</th>
<th>Patient age (years)</th>
<th>Tumour volume (cm³)</th>
<th>Serum ALP at diagnosis (U/l)</th>
<th>11β-HSD1 immunostaining (0–4 scale)</th>
<th>11β-HSD2 immunostaining (0–4 scale)</th>
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<td>Osteoblastoma with epithelioid features</td>
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<td>Osteoblastoma with pseudomalignant features</td>
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<td>Osteoblastoma</td>
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ALP, alkaline phosphatase; NEG, negative; OS, osteosarcoma.

Table 1 Semiquantitative analysis of glucocorticoid-modifying enzyme expression in malignant and non-malignant tumours arising from osteoblasts (pre-treatment biopsy specimens). Enzyme expression was assessed semiquantitatively in a blinded fashion on a scale of 0–4, where 0, indicated no expression; 1, low expression; 2, medium expression; 3, high expression and 4, very high expression.
that high 11β-HSD1 expression in OS tissue in vivo is associated with smaller tumour volume.

The ability of 11β-HSD2 to inactivate endogenous glucocorticoids has been proposed as the mechanism by which 11β-HSD2 has a stimulatory effect on tumour proliferation (Rabbitt et al. 2002, 2003). An association of 11β-HSD2 expression with malignancy or glucocorticoid resistance has also been reported in OS cells, pituitary tumours, adrenal tumours and leukaemic cells (Coulter et al. 1998, Rabbitt et al. 2002, 2003, Sai et al. 2011). Expression of 11β-HSD2 in OS tissue will render it insensitive to endogenous glucocorticoids such as cortisol. The finding that OS 11β-HSD2 enzyme expression could be used to activate, rather than inactivate, specific synthetic glucocorticoids suggests that any pro-tumour effect of 11β-HSD2 could be converted to an anti-tumour effect. The synthetic glucocorticoid chosen in this study has the advantage that its inactive form is generated to a small extent in vivo during treatment with DEX (Best et al. 1997). The lack of any harmful effects of this inactive glucocorticoid on healthy tissue makes it a good candidate to be taken forward for further study as a possible anti-cancer therapeutic. We therefore hypothesise that systemic treatment with DHD would have minimal effect on cells that lack 11β-HSD2, as DHD is unable to activate the glucocorticoid receptor. However, 11β-HSD2 expression in OS tissue would cause the intracellular generation of DEX from DHD. Some DEX would also be regenerated by endogenous 11β-HSD2 that is primarily found in the kidney. However, this is unlikely to be a problem as renal tubular cells do not appear to be detrimentally affected by DEX, and DEX is unable to bind to mineralocorticoid receptors and so would not cause hypertension (Cooper & Stewart 1998). Although we have evaluated DHD, it is possible that structurally related synthetic glucocorticoids could be generated that have even greater ability to be activated powerfully and selectively by the 11β-HSD2 enzyme. Previous reports have analysed some of the structural determinants that determine directionality of glucocorticoid conversion by the 11β-HSD enzymes (Diederich et al. 2002) and this should facilitate development of additional glucocorticoids.

An interesting observation within our study was the relative difference in the directionality of 11β-HSD2 for synthetic glucocorticoids between the MG-63 OS and transfected HEK cell line. The most likely explanation for this is the dependence of the 11β-HSD2 enzyme on the intracellular NADH/NAD+ ratio. NADH is an essential cofactor for the reductase reaction with synthetic glucocorticoids.

**Figure 2** (A) Correlations between 11β-HSD enzyme expression in pretreatment osteosarcoma tissue biopsies and serum alkaline phosphatase at diagnosis (n=11). The expression of 11β-HSD1 and 11β-HSD2 were assessed semiquantitatively in a blinded fashion on a scale of 0–4, where 0, indicated no expression; 1, low expression; 2, medium expression; 3, high expression and 4, very high expression. (B) Correlations between 11β-HSD enzyme expression in pretreatment osteosarcoma tissue biopsies and estimated primary tumour volume (n=10).
The NADH/NAD$^+$ ratio is known to be elevated in various cancers (Bui & Thompson 2006) and the ratio is likely to be variable depending on the origin of the cell and the rapidity of its proliferation.

A limitation of our study is the problem of obtaining large amounts of OS tissue before chemotherapy. Pretreatment diagnosis of OS is typically based on the microscopic findings in needle biopsies, most of which has to be used for diagnostic purposes. As such, we were unable to examine quantitatively the relationship between 11β-HSD2 expression in pretreatment biopsies and the subsequent degree of tumour necrosis in response to chemotherapy. We were, however, able to perform a semiquantitative analysis of protein expression by immunohistochemistry and relate this to tumour characteristics known to have an impact on patient prognosis. This analysis demonstrated that both 11β-HSD1 and 11β-HSD2 were expressed but their expression was highly variable with variability greater for 11β-HSD1 than 11β-HSD2. Several factors are known to regulate 11β-HSD1 expression in non-transformed osteoblasts. These include age, pro-inflammatory cytokines and glucocorticoids themselves (Cooper et al. 2001, 2002). In this small sample, 11β-HSD1 expression was not associated with age. It is possible that factors such as the local immune response could impact on 11β-HSD1 and therefore the rate of tumour proliferation, but this would need to be examined in further studies.

An issue potentially limiting the ability to use glucocorticoids such as DHD as an OS therapy is the almost universal use of high doses of DEX during chemotherapy. Although not part of the formal chemotherapy regimen, high doses of DEX are used for its anti-emetic properties. It will be interesting to examine the extent to which this use of DEX is related to beneficial responses to chemotherapy. Given the

**Figure 3** (A) Correlations between 11β-HSD enzyme expression and response to chemotherapy. The mRNA expression and enzyme activity for 11β-HSD1 and 11β-HSD2 were assessed relative to the degree of necrosis measured in post-chemotherapy samples ($n=22$). A level of necrosis of $<90\%$ indicates a poor prognosis with osteosarcoma. (B) The correlation between mRNA expression for 11β-HSD enzymes and alkaline phosphatase (a marker of osteoblasts/osteosarcoma tissue) was also examined ($n=11$).
common use of glucocorticoids in the OS chemotherapy regimen, it is likely that inactive glucocorticoids will have most potential in treating patients with disease relapse. These patients have a poor prognosis using current treatment approaches and, as such, there is a need for novel therapeutic approaches in this setting.

We have demonstrated that OS tissue is capable of metabolising glucocorticoids and that 11β-HSD2-mediated glucocorticoid inactivation appears to be associated with tumour aggressiveness. However, glucocorticoids that lack biological activity in normal tissue can be selectively activated in OS tissue by the same enzyme. The development and use of glucocorticoids that are selectively activated by the 11β-HSD2 enzyme could form the basis of new therapies for OS.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

This study was funded by a grant from the University Hospital Birmingham Charities.

**Acknowledgements**

We would like to thank Maurice Adkins, Hamed Ali, Elaine Haywood and Karen Joynes from the Department of Musculoskeletal Pathology, Royal Orthopaedic Hospital, Birmingham for their technical assistance.
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Received in final form 3 June 2012
Accepted 19 June 2012
Made available online as an Accepted Preprint 20 June 2012