Multidrug Resistance and Malignancy in Human Osteosarcoma

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ABSTRACT

In osteosarcoma, resistance to chemotherapy and metastatic spread are the most important mechanisms responsible for the failure of current multimodal therapeutic programs. We have shown previously that overexpression of the MDRI gene product P-glycoprotein is the most important predictor of an adverse clinical course in patients with osteosarcoma treated with chemotherapy. In this study, we analyzed the relationship between P-glycoprotein expression and local aggressiveness and systemic dissemination of multidrug-resistant (MDR) human osteosarcoma cells. Compared to parental sensitive cells, MDR cells showed a decreased tumorigenicity and metastatic ability in athymic mice, together with a reduced migratory and invasive ability and a lower homotypic adhesion ability in vitro, suggesting that P-glycoprotein overexpression is associated with a less malignant phenotype. These experimental observations were confirmed by clinical data. In fact, the time of appearance of lung metastases in a series of osteosarcomas patients treated with chemotherapy was significantly shorter in the group of cases with no expression of P-glycoprotein in the primary lesion compared to the group with P-glycoprotein overexpression. Moreover, the incidence of P-glycoprotein overexpression was found to be higher among patients with localized disease at the clinical onset than in patients with evidence of metastasis at the time of diagnosis. These data indicate that, in osteosarcoma, the MDR phenotype is not associated with a more aggressive behavior both in vitro and in clinical settings, suggesting that the previously shown association of the MDR phenotype with a worse outcome in osteosarcoma is not related to a higher metastatic ability of cells with P-glycoprotein overexpression but is more likely due to their lack of responsiveness to cytotoxic drugs.

INTRODUCTION

The occurrence of resistance against cytotoxic agents is a major problem in cancer chemotherapy. Among the different mechanisms of drug resistance, the best known is MDR, which is commonly associated with the expression of P-glycoprotein, a Mr 170,000 transmembrane energy-dependent efflux pump encoded by the MDRI gene (1). Overexpression of P-glycoprotein enables cancer cells to circumvent the lethal effects of a wide range of anticancer drugs (2). Although it is now well established that P-glycoprotein plays a central role in MDR in vitro (3), its clinical relevance has not been completely defined in most human neoplasms (4-6). Clinical studies on different malignant tumors have shown the progressive development of P-glycoprotein overexpression during chemotherapy (7, 8), confirming the hypothesis that in clinical conditions the exposure to antineoplastic agents may select MDR clones whose emergence may ultimately result in the relapse of the disease. Immunostaining analysis of P-glycoprotein expression has revealed that moderate to high levels of this protein may be present at the time of diagnosis, before chemotherapy, in a number of neoplasms, such as leukemia (9, 10), lymphoma (5), colon carcinoma (4), neuroblastoma (11), and sarcomas (12-16), including osteosarcoma (17, 18), and that P-glycoprotein may have a prognostic value. Among solid tumors, a relationship between P-glycoprotein overexpression and an adverse clinical course has been observed in neuroblastoma (11), childhood soft tissue sarcomas (15), and osteosarcoma (19). However, the biological mechanisms responsible for the adverse role of P-glycoprotein in the outcome of these tumors is still unclear, and in particular, it is not yet determined whether the prognostic value of P-glycoprotein overexpression simply reflects the lack of effectiveness of chemotherapy on MDR cells or is related to a higher degree of malignant potential. Studies of multistage liver carcinogenesis in animal tumor models (8) as well as clinical data on different human neoplasms support the hypothesis that P-glycoprotein may be associated with a more malignant phenotype. In neuroblastoma, P-glycoprotein expression correlates with the stage of the tumor (11). In colon carcinoma, P-glycoprotein is associated with an increase in local tumor aggressiveness (20). In osteosarcoma, the incidence of P-glycoprotein overexpression is higher in metastatic than in primary lesions (18), and P-glycoprotein overexpression is significantly associated with a higher risk of relapse (19). These findings, together with the documented increased expression of the MDRI gene in tumors derived from tissues that normally do not express the P-glycoprotein (2), indicate the possible association of P-glycoprotein expression with tumor progression. On the contrary, several studies have suggested an inverse relationship between P-glycoprotein expression and malignant phenotype in experimental systems (21, 22). Therefore, the association of MDR with progression appears to be an intriguing and still incompletely defined phenomenon. In this study, we analyzed the invasive, tumorigenic, and metastatic characteristics of MDR human osteosarcoma cells as well as the different pattern of metastatic disease in a clinical series of patients with osteosarcoma with respect to the expression of P-glycoprotein to establish the malignant potential of MDR cells in this tumor.

MATERIALS AND METHODS

Cell Lines. Starting from the human osteosarcoma cell line U-2 OS, stepwise increases in doxorubicin concentration produced variants that were resistant to 30 ng/ml doxorubicin (referred to as U-2 OS/DX30), to 100 ng/ml doxorubicin (U-2 OS/DX100), and to 580 ng/ml doxorubicin (U-2 OS/DX580), respectively (23). U-2 OS cells were maintained in Iscove’s modified Dulbecco’s medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml; Life Technologies, Inc., Paisley, Scotland), and 10% heat-inactivated FCS (Biological Industries, Kibbutz Beth Haemek, Israel) at 37°C in a humidified 5% CO2 atmosphere. MDR variants were continuously cultured in the presence of their corresponding concentrations of doxorubicin. All of the MDR cell lines here considered showed the typical in vitro drug sensitivity pattern of the MDR phenotype, consistent with a P-glycoprotein-mediated mechanism, as shown by an increased expression of the MDRI gene without either an altered glutathione-S-transferase activity (23) or an impaired topoisoerase II activity. Doubling time was determined by daily harvesting of cells after seeding of...
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20,000 cells/cm². Cell viability was determined by trypan blue dye exclusion. For the analysis of differentiation features, cells were harvested 96 h after seeding and smeared on glass slides for avidin-biotin immunocytochemistry. Cytopsins were fixed with methanol:acetone (3:7) at -20°C for 10 min. The following polyclonal antibodies were used: anti-osteonectin LF-bONTI (1:200), anti-osteopontin LF-19 (1:100), anti-osteocalcin LF-32 (1:200), and anti-bone sialoprotein LF-6 (1:100), all kindly provided by Dr. L. W. Fisher (Bone Research Branch, NIH, Bethesda, MD).

Chemotaxis Assay. The chemotaxis assay was carried out using Transwell chambers (Costar, Cambridge, MA) as described previously (24). Briefly, 1.5 x 10⁶ cells were resuspended in DMEM (Life Technologies, Inc.) containing 0.1% BSA (Sigma Chemical Co., St. Louis, MO) and seeded in the upper compartment of the chamber. A 24-h supernatant from BALB/c 3T3 fibroblasts (Nucleopore, Pleasanton, CA) coated with 5 µg/ml gelatin (Sigma). Cells were allowed to migrate for 6 h at 37°C. After the incubation, cells that had migrated to the lower side of the filter were fixed in ethanol and stained with toluidine blue. Five to 10 fields/filter were counted at X160. Three different experiments were made for each cell line.

Chemoinvasion Assay. This assay is a modification of the chemotaxis assay (25). Polycarbonate filters coated with Matrigel (16.5 µg/filter; Collaborative Biomedical Products, Bedford, MA) were placed in chemotaxis chambers. After 6 h of incubation, invaded cells on the lower surface were scored as for chemotaxis. Three different experiments were made for each cell line.

Homotypic Adhesion Assay. Homotypic adhesion assay was performed as described previously (26). Briefly, 2 ml of a 10⁶ cells/ml unincellular suspension were incubated at 37°C for 60 min. At the end of the incubation, cells were resuspended with a large-bore Pasteur pipette. Homotypic adhesion was then evaluated microscopically by counting single cells at the end of the procedure.

Tumorigenic and Metastatic Ability in Athymic Mice. Female athymic 4-5-week-old Crlnu/hnu (CD-1) BR mice (Charles River Italia, Como, Italy) were used. Tumorigenicity was determined after s.c. injection of 30 x 10⁶ cells. Tumor growth was assessed twice weekly. Mice were sacrificed 3-6 months after inoculation. The metastatic ability of parental and resistant cell lines was determined by injection of 2 x 10⁶ viable cells in a tail vein. To obtain natural killer-depressed animals, some groups of mice were injected i.v. with 0.4 ml of a 1:25 dilution of anti-asialo GM₁ antiseraum (Wako, Düsseldorf, Germany) 24 h before cell inoculation. Two months later, mice were sacrificed, and the number of pulmonary metastases was determined by counting with a stereomicroscope after staining with black India ink. Histological sections obtained from the tumors grown in athymic mice were stained with H&E.

Adhesion Molecules and Integrin Pattern. The expression of ICAM-1, LFA-3, and A-CAM adhesion proteins as well as αβ(1), αβ(2), αβ(3), and αβ(4) integrins was determined by flow cytometry (FACScan; Becton Dickinson, San Jose, CA) after indirect immunofluorescence with the following monoclonal antibodies: LFA-3 (anti-LFA-3, Immunotech S.A.); CD54 ICAM (anti-ICAM-1; Immunotech S.A.); GC-4 (anti-A-CAM; Sigma); CDw49b VLA2 (anti-α 2 chain, a2ß₃; Immunotech S.A.); CDw49d VLA4 (anti-α 4 chain, a4ß₃; Immunotech S.A.); and CDw49f VLA6 (anti-α 6 chain, a6ß₄; Immunotech S.A.).

Table 1 Experimental metastatic ability of U-2 OS osteosarcoma cell line and of its MDR variants in immunodepressed mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Lung colonies in nude mice</th>
<th>Lung colonies in anti-asialoGM₁ treated mice</th>
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<tbody>
<tr>
<td>U-2 OS</td>
<td>8/10</td>
<td>2/10</td>
</tr>
<tr>
<td>U-2 OS/DX⁵⁰</td>
<td>ND⁵</td>
<td>ND⁵</td>
</tr>
<tr>
<td>U-2 OS/DX¹⁰⁰</td>
<td>ND⁶</td>
<td>ND⁶</td>
</tr>
<tr>
<td>U-2 OS/DX⁵⁸⁰</td>
<td>0/10⁴</td>
<td>0/10⁴</td>
</tr>
</tbody>
</table>

5 ND, not done.
6 P < 0.05.
7 P < 0.001.

Clinical Series. The present study was performed on a series of patients with newly diagnosed osteosarcoma seen at the Rizzoli Institute in Bologna between September 1986 and December 1989. The clinical and pathological characteristics of the 92 patients considered eligible for a clinical study aimed at the optimization of chemotherapy for osteosarcoma (27) and analyzed for their P-glycoprotein expression have been described in a previous paper (19). In this series of patients with localized high-grade osteosarcoma of the extremities treated with the same regimen of multiple drug chemotherapy, the interval from initial diagnosis and metastatic relapse was considered here. We also analyzed the P-glycoprotein status of 18 patients referred to the Rizzoli Institute during the same period and excluded from the previous study because of evidence of metastases at the onset. P-glycoprotein immunostaining was performed on tumor samples obtained before chemotherapy, as described previously (19). Briefly, 5-µm sections from undecalcified, formalin-fixed, paraffin-embedded tissue samples were immunostained by avidin-biotin complex immunoperoxidase using JSB-1 monoclonal antibody (Sanbio, Uden, the Netherlands) diluted 1:20 as primary antibody. Tissue sections from normal kidney were used as positive control.

Statistical Analysis. Fisher’s exact test (28) was used to evaluate the association between two dichotomous variables.

RESULTS

In Vivo Studies. U-2 OS-resistant variants (U-2 OS/DX⁵⁰, U-2 OS/DX¹⁰⁰, and U-2 OS/DX⁵⁸⁰) showed progressive increased levels of MDR (15-, 58-, 330-fold, respectively) and a parallel progressive increase in P-glycoprotein expression (23). After inoculation into athymic mice, all of the MDR variants showed a different pattern of tumorigenic and metastatic ability compared to the parental cell line. In fact, the incidence of experimental metastases was progressively lower in MDR cell lines according to the level of resistance, and U-2 OS/DX⁵⁸⁰ cells were completely unable to give pulmonary colonies after i.v. injection both in untreated and in anti-asialo GM₁-pretreated mice (Table 1). A similar trend was observed with regard to the tumorigenic potential, as shown by the complete lack of ability of U-2 OS/DX⁵⁸⁰ to produce tumors after s.c. inoculation (Table 2).

In Vitro Studies. In the MDR osteosarcoma cell lines, the observed low metastatic and tumorigenic potential was not associated with differences in the in vitro growth pattern, as indicated by the presence of a similar doubling time in parental and MDR cells, but was more likely related to a higher level of differentiation (Table 3). In fact, MDR variants showed a significantly higher expression of osteopontin and osteocalcin, two markers of the osteoblastic lineage (29). Moreover, the in vivo data are in agreement with a reduced in vitro chemotactic and invasive ability and with a reduced homotypic adhesion potential. In fact, compared to U-2 OS cells, the number of cells migrating after a chemotactic stimulus (Fig. 1), the number of cells migrating through Matrigel-coated filters (Fig. 2), and the formation of cell clumps in a homotypic adhesion assay (Fig. 3) were all significantly lower in the MDR variants.

To determine whether the in vivo and in vitro behavior of MDR cells was associated with modifications of their plasma membrane, we evaluated the expression of some adhesion proteins and integrins. No significant difference was observed between U-2 OS and its MDR variants with regard to ICAM-1, LFA-3, and A-CAM profiles (data not shown), whereas the expression of αβ(1), αβ(2), αβ(3), and αβ(4),...
Table 3 In vitro cell growth and immunocytochemical expression of osteogenic differentiation markers of U-2 OS osteosarcoma cell line and of its MDR variants

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Doubling time (h)</th>
<th>Expression of osteogenic differentiation markers&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ON&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>U-2 OS DX&lt;sup&gt;30&lt;/sup&gt;</td>
<td>20.3 ± 2.8</td>
<td>57</td>
</tr>
<tr>
<td>U-2 OS DX&lt;sup&gt;100&lt;/sup&gt;</td>
<td>26.5 ± 2.3</td>
<td>55</td>
</tr>
<tr>
<td>U-2 OS DX&lt;sup&gt;580&lt;/sup&gt;</td>
<td>28.2 ± 2.3</td>
<td>52</td>
</tr>
<tr>
<td>U-2 OS DX&lt;sup&gt;380&lt;/sup&gt;</td>
<td>26.4 ± 3.2</td>
<td>54</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are expressed as percentage of positive cells of 300 cells.

<sup>b</sup>ON. osteonectin; OP. osteopontin; OC. osteocalcin; BSP. bone sialoprotein.

<sup>c</sup>P < 0.001.

Fig. 1. In vitro chemotactic ability of U-2 OS-sensitive cell line and of its MDR variants. A 24-h supernatant from WT-BALB/c 3T3 cells was used as a source of chemoattractant. Each column represents the mean of three independent experiments; bars. SD. DX/30. U-2 OS/DX30 cells; DX/100. U-2 OS/DX100 cells; DX/580. U-2 OS/DX580 cells. *, P < 0.001.

No. of migrated cells/field

![Graph](image-url)

No. of cells migrated/field

![Graph](image-url)

Percentage of aggregated cells

![Graph](image-url)

Discussion

Although the phenomenon of MDR has been extensively analyzed during the last two decades, there are still several aspects of the biology of resistant cells, distinct from their reduced susceptibility to the cytotoxic effects of anticancer agents, that deserve further investigation. In particular, only a few, partially conflicting data have been reported on the relationship between P-glycoprotein expression and aggressiveness and metastatic potential of MDR cells. Several reports have shown changes of the tumorigenic and metastatic ability of resistant cells after the acquisition of the MDR phenotype (21, 22, 30, 31), suggesting the existence of an inverse relationship between MDR and the malignant potential. However, a study analyzing the expression of P-glycoprotein during stepwise rat liver carcinogenesis (8), as well as clinical data investigating the significance of P-glycoprotein expression in human malignant tumors, have supported the hypothesis that P-glycoprotein overexpression is related to a highly malignant phenotype. In fact, in addition to the finding that in many solid tumors P-glycoprotein expression is generally increased after chemotherapy (7), in colon carcinoma the presence of P-glycoprotein-positive cells has been found to be associated with a greater incidence of vessel invasion and lymph node metastasis (20); in neuroblastoma, P-glycoprotein expression has been correlated with the stage of the tumor, and its level has been found to be higher in metastatic than in primary tumors (11). With regard to solid tumors of mesenchymal derivation, P-glycoprotein overexpression has been shown to be an important adverse prognostic marker in childhood soft tissue sarcoma (15) and in osteosarcoma (19). These findings, suggesting a positive association of P-glycoprotein expression with tumor progression, conflict with the conclusions drawn from the in vitro conditions. The discrepancy observed between clinical and experimental findings indicates the existence of a complex relationship between MDR and metastasis and suggests the need for a direct comparison of in vitro and clinical data in the same model.
In this study, we analyzed the association of P-glycoprotein expression with local aggressiveness and dissemination in osteosarcoma both \textit{in vitro} and in a clinical series of patients. In this highly malignant primary bone tumor, the use of chemotherapy in addition to surgery has significantly improved the outcome (27, 32), but despite an increase in the survival rate, a considerable proportion of patients still develop metastases and die of their disease. Resistance to antineoplastic agents appears to be critical for the outcome of osteosarcoma (33). The presence of P-glycoprotein in primary untreated tumors (17–19), the higher expression of P-glycoprotein among the metastatic lesions compared to primaries (18), and the highly significant prognostic value of P-glycoprotein (19) may suggest the existence of an association of MDR with tumor progression in osteosarcoma.

However, in this study, we have observed a decreased tumorigenic and metastatic ability in MDR osteosarcoma cells compared to parental cells. The malignant potential was found to be inversely related to the MDR level, with U-2 OS/DX$^{580}$ being completely unable to produce tumors at the site of injection or to give pulmonary metastases. This change of the \textit{in vivo} growth was not due to an altered \textit{in vitro} growth pattern but can be attributed to the acquisition of a more
differentiated phenotype, as shown by the significant increase in the expression of osteopontin and osteocalcin, two markers of osteoblastic differentiation. Moreover, the lower tumorigenic and metastatic ability of MDR cells appeared to be correlated with a decrease of in vitro chemotactic and invasive potential as well as with a lower homotypic adhesion ability. These findings raise the possibility that, in osteosarcoma cells, P-glycoprotein overexpression is associated with a more differentiated phenotype and with altered cell adhesion characteristics, and that, in turn, these changes may influence cancer dissemination. The process of tumor invasion and metastasis involves changes of cell-to-cell and cell-to-substratum interactions (34). The analysis of some adhesion proteins and β1 integrins revealed a progressive increase in the expression of α5β1, αvβ3, αvβ5, and αvβ6 integrins together with the level of MDR, whereas a remarkable change was observed with regard to ICAM-1, A-CAM, and LFA-3 proteins. A higher expression of integrins, as well as a higher expression of bone matrix proteins, may contribute to the immobilization of tumor cells to the surrounding extracellular matrix, therefore inhibiting early events of the metastatic cascade. However, our data did not establish a cause-effect relationship between changes of integrin pattern and modifications of the in vivo behavior of malignant cells but only added these proteins to the long list of plasma membrane proteins, the expression of which is altered in association with MDR (21, 35).

A decrease in tumor aggressiveness and malignancy in association with MDR had already been observed in different experimental models (21–22, 31). In osteosarcoma we could substantially confirm our experimental finding by the analysis of a clinical series. Apart from the well-known general favorable effect of adjuvant chemotherapy on the relapse-free survival in patients with osteosarcoma, a changed pattern of relapse, most often involving pulmonary metastases, has been observed in this tumor after the adoption of adjuvant chemotherapy programs. In particular, a smaller number of metastatic lesions appearing after a longer disease-free interval has been observed with intensive chemotherapy (32, 36, 37). Moreover, additional second-line chemotherapy has little if any effect on these patients, suggesting that they are resistant to further multidrug protocols (37). In this study, we analyzed a group of patients treated with the same chemotherapeutic regimen and stratified for the P-glycoprotein expression. In this series, the relapse rate had been found previously to be significantly higher in cases overexpressing P-glycoprotein than in P-glycoprotein-negative cases (19), but among the relapsed patients, metastases were found to occur earlier in P-glycoprotein-negative than in P-glycoprotein-overexpressing cases. Moreover, by analyzing the expression of P-glycoprotein in a group of osteosarcoma patients with metastases at the onset, we found that the percentage of cases overexpressing P-glycoprotein in their primary lesion was lower in this group compared to those with localized disease. Although this observation must be confirmed by the analysis of a larger series of patients, it appears that, in osteosarcoma, P-glycoprotein overexpression is not associated with a higher aggressive phenotype but might even be associated with a lower malignancy potential.

In conclusion, taken together, our data suggest that in osteosarcoma the significant association of the MDR phenotype with a less favorable clinical outcome (19) is not caused by a concomitant increase in the ability to invade and metastasize. Whether the difference is really due to clinical drug resistance mediated by P-glycoprotein obviously remains to be proved. We propose that P-glycoprotein-overexpressing osteosarcoma cells, being resistant to a variety of drugs, including doxorubicin, the most active agent against osteosarcoma (38), may be selected during chemotherapy and slowly, but with a high probability, lead to the relapse of the tumor. This hypothesis seems to be confirmed by the observed higher incidence of P-glycoprotein overexpression among metastases of osteosarcoma developing during or after chemotherapy compared to primary, untreated tumors (18), probably reflecting the emergence of MDR subpopulations during the treatment. The prognostic value of P-glycoprotein as an adverse marker of outcome in osteosarcoma appears to be statistically significant only on long-term follow-up (19), suggesting that in this tumor the “less malignant” MDR cells require a longer period of time to become clinically evident as pulmonary metastases. However, at the present time, the possibility cannot be excluded that P-glycoprotein overexpression may be associated with other, still undefined factors, leading to a less favorable response to therapy. For example, it is possible that exposure to cytotoxic agents causes activation or inactivation of genes involved in the modulation of tumor aggressiveness (39). Moreover, it is impossible to determine whether P-glycoprotein overexpression shows a cause-effect association with the reduced metastatic potential of MDR cells or rather reflects a more complex phenotype. MDR gene transfection studies may help to clarify this interesting problem. In any case, if confirmed in a larger series, the finding that in osteosarcoma the significant association of the MDR phenotype with a less favorable clinical outcome is not related to a higher metastatic ability might have relevant clinical implications, because it would further support the potential value of resistance modifiers for osteosarcoma patients with P-glycoprotein overexpression in their primary tumor at the time of diagnosis.

REFERENCES


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