Introduction: It is generally accepted that neoplasia develops through a multistep process involving the acquisition of genetic alterations leading to malignancy. Benefits that would accrue from a more thorough understanding of these genetic events may include better patient management. For example, the treatment of osteosarcoma is limited by the lack of prognostic markers to identify patients with a poor prognosis after conventional therapy; identification of the genes involved in tumor initiation and progression may help to identify this important patient subgroup. Although alterations of a number of genes have been shown to be involved in osteosarcoma, their relationship to stages of tumor progression and actual disease outcome remains unresolved. We therefore elected to examine osteosarcoma samples in order to identify genes involved in tumor progression (through the analysis of gene expression).

One known gene which was isolated was von Willebrand factor. Very few patients with osteosarcoma who develop metastases will be cured. The process of hematogenous tumor cell metastasis involves a number of distinct steps, of which neovascularization and platelet aggregation are critical. These two processes both involve von Willebrand factor (vWF), a large glycoprotein expressed exclusively by endothelial cells and megakaryocytes. It is involved in the platelet adhesion, aggregation and interaction with the subendothelial matrix during coagulation (through the interaction of vWF and platelet specific vWF receptors). Inappropriate expression of the vWF gene may play a critical role in the metastatic spread of osteosarcoma. We therefore chose to investigate the role of vWF in osteosarcoma through the analysis of mRNA and protein expression in tumor samples.

Methods: 1) Tumor Samples: osteosarcoma tumor samples were obtained immediately following surgical resection and frozen in liquid nitrogen to prevent RNA degradation. The RNA was extracted using standard procedures and quantitated by both spectrophotometric determination and quantitative reverse-transcriptase PCR (RT-PCR). Samples were obtained at different stages of tumor progression (at biopsy, and/or resection and metastasis) from 5 patients (12 tumor samples). In addition, 3 high grade tumors that did not metastasize, 4 low grade tumors, 7 metastatic samples, 1 osteoblast cell culture, and 8 tumor cell lines were analyzed. 2) Differential Display and Sequencing: differential display was performed essentially as described by Liang et al (Science, 257:967, 1992) and Bauer et al (Nucleic Acids Research, 21:4272, 1993) except that the PCR was performed using a range of cycles. CDK4 expression was used as a positive control. Differentially expressed bands, excised from the DDPCR gels, were re-amplified before being directly sequenced using the Amersham Thermosequenase kit. 3) vWF RT PCR Quantitation: Total cellular RNA was reversed transcribed into cDNA by reverse transcriptase (MMLVRT) and amplified for vWF and asparagine synthetase. The vWF and AS RNA specific primers were chosen to amplify a region containing at least one intron, thereby minimizing the effects of contaminating DNA. A range of PCR was examined for each sample to ensure that the reaction was in the logarithmic phase of amplification (when the amount of product corresponds to the amount of the initial template). Asparagine synthetase (AS) was co-amplified along vWF in order to act as an internal control for the PCR reaction. The products were photographed for quantitative densitometry using a Molecular Dynamics densitometer. 4) Immunohistochemistry: formalin fixed paraffin embedded tissue sections were stained with anti-vWF antibody and counterstained with hematoxylin.

Results: In order to isolate genes involved in tumor progression, 19 DDPCR primer pairs were used to examine 26 osteosarcoma samples of different stage and grade. Eight genes, including 5 known and 3 novel genes, were isolated and confirmed by quantitative PCR to be differentially expressed. One of the genes, isolated on the basis of higher expression in metastatic samples of paired primary and metastatic specimens from the same patient, was von Willebrand factor. Quantitative reverse-transcriptase PCR confirmed that vWF was more highly expressed in metastatic samples compared with the corresponding paired primary tumor in all 5 cases. In addition, vWF was found to be expressed at a higher level in metastatic samples compared with unmatched primary samples (p=0.05). In addition, vWF expression was detected in the SAOS2 osteosarcoma cell line. Immunohistochemistry was performed on 15 samples in order to determine the source of the differential gene expression. The majority of gene expression resulted from vascular cells. However, in 3 cases, tumor cells stained positively for vWF, including the 2 samples with highest vWF expression. In addition, of one paired primary/metastatic pair, only the metastatic sample stained positively for vWF.

Discussion: The differential expression of vWF in metastatic samples and the immunohistochemistry results suggests that vWF and the vWF receptors may play a role in osteosarcoma metastasis. The expression of vWF in osteosarcoma tumor samples may result from two sources: the tumor cells themselves and the endothelial cells present in the vasculature of the tumor. The immunohistochemistry study indicates that the majority of differential expression may be due to endothelial cells in the vasculature. vWF staining of endothelial cells is commonly used as a marker for vascularity in tumor sections. The amount of vascularule, referred to as vascular index or microvessel density, has been shown to be of prognostic value in cancers such as breast, melanoma, prostate, ovarian, gastric, and colon carcinoma. This may also be the case in osteosarcoma. Thus the differential expression of vWF may be partially due to differences of microvessel density in the tumor samples.

In addition to the expression of vWF by endothelial cells present in the tumor, vWF was shown to be expressed by the tumor cells themselves in three tumors. This is the first reported case of vWF expression by tumor cells clearly not of vascular origin. The vWF expression by tumor cells may play a role in the aggressive metastatic spread of osteosarcoma. The aggregation of platelets and tumor cells may result in metastasis by producing a mixed platelet-tumor cell thrombus which can then easily lodge in a capillary or arteriole. It has been shown that tumors capable of forming platelet aggregates usually metastasize to the lung, the first sub-vasculature a metastatic cell would encounter in the blood stream, while those lacking this ability gave a more widespread occurrence in mice. This tumor cell-platelet mechanism may be partially responsible for metastasis of osteosarcoma (which tend to metastasize to the lung). In support of this, it has been reported that the osteosarcoma cell lines MG63, HOS and SAOS2 (which we show expressed vWF) induced platelet aggregation. Pre-treatment of either tumor cells or platelets with an antibody or peptide which neutralizes vWF or blocks vWF receptor binding has been shown to inhibit tumor cell-platelet interaction in vitro and tumor cell metastases in vivo (in mice) for both colon carcinoma and melanoma cell lines. In addition, it has been reported that vWF receptors are involved in the platelet aggregation induced by the SAOS2, HOS and MG63 osteosarcoma cell lines.

The analysis of vWF mRNA and protein expression in osteosarcoma specimens has indicated that it is expressed more highly in metastatic samples. In addition, osteosarcoma cells expressed vWF in three cases. This study has highlighted two processes which may be important for the spread of osteosarcoma: angiogenesis and the involvement of platelet aggregation in metastasis. Further analysis of a possible correlation between microvessel density and prognosis is presently underway, as are studies into the mechanism of platelet aggregation in osteosarcoma metastasis.

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