Mechanism of Osteosarcoma Tumorigenesis by S100A4

INTRODUCTION

Osteosarcoma (OS) is the most common primary malignancy of bone in children and young adults. Although current treatment protocols involve both wide local resection and chemotherapy, the average five-year disease free survival is only 50-65%. Despite significant advances in surgical techniques and chemotherapeutic regimens over the past few decades, there has been minimal improvement in patient survival. Therefore, there is a critical need to identify both molecular markers of OS tumor progression, and molecular targets for therapeutic intervention.

The S100 family of proteins contains 20 members of low molecular weight calcium binding proteins located on chromosome 1. Many of these proteins, including S100A2, A4 and A7, have found to be differentially expressed in human tumors. Furthermore, these proteins have been linked to cellular tumor-promoting effects leading to the development of different cancers. Previous studies have shown that S100A4 expression is associated with OS tumorigenesis, and we have found a high endogenous level of S100A4 expression in the metastatic MG63.2 OS cell line when compared to the less metastatic MG63 parental line. In this study, we elucidate the mechanism underlying OS tumorigenesis by S100A4.

METHODS

We used RT-PCR to measure the endogenous levels of S100A4 in three different OS cell lines. S100A4 was then overexpressed by adenoviral (Adv) transduction or knocked down with siRNA in three different OS cell lines (MG63, MG63.2, and 143B). Adv-RFP was used as a negative control. We then characterized Adv-transduced OS cells in vitro for cell proliferation, migration, invasion, apoptosis, and osteogenic differentiation. Cell proliferation was assayed using Trypan Blue cell counting and Crystal Violet staining. Doubling times were calculated as previously reported. Cell migration and invasion were measured using previously described Wound Healing and Matrigel assays, respectively. For the wound healing assay, a scratch was made in a confluent cell monolayer, and the ability of adenovirally transduced OS to close this gap after 36 hours was measured. For the Matrigel assay, OS cells were plated in the upper chamber of a transwell unit, and assayed for their ability to invade a Matrigel matrix towards a serum chemoattractant. Apoptosis was determined using immunohistochemical staining for the late apoptotic marker Caspase 3 and flow cytometry with Annexin-V and Propidium Iodide. Cells were further categorized as alive, early apoptotic, or late apoptotic. Finally, osteogenic differentiation was measured with immunohistochemical staining for both early [alkaline phosphatase (ALP)], and late [osteocalcin (OCN), osteopontin (OPN), and Alizarin Red] markers of osteogenic differentiation. We measured the ability of S100A4 and siS100A4 to induce osteogenic differentiation in both OS cell lines and the Mesenchymal Stem Cell line C3.H10.

Adv-transduced OS cells were then then injected subperiosteally into the proximal posterior tibia of athymic nude mice, and we measured the ability of OS cells to form primary tumors. Tumor size was measured every 3-4 days and the tumor volume and doubling time were calculated as previously reported.

RESULTS

Expression levels of S100A4 were significantly increased in the metastatic MG63.2 OS cell line when compared to the parental (less-metastatic) MG63 OS cell line. S100A4 adenoviral overexpression and siRNA knockdown in OS cells was confirmed with RT-PCR and Western Blot. Exogenous expression of S100A4 promoted cell proliferation in vitro (P<0.03) and decreased doubling times (P<0.04). Conversely, S100A4 knockdown inhibited cell proliferation and increased doubling times. Cell invasion through a Matrigel matrix was inhibited by S100A6 knockdown (p<0.02), while S100A6 overexpression correlated to significantly greater invasive potential (p<0.02). S100A6 overexpression also resulted in increased cell migration in a Wound Healing Assay (p<0.001). Mechanistically, S100A4 prevented cell cycle arrest and inhibited the expression of early apoptotic markers after the cells were serum starved. Conversely, S100A4 knockdown induced the expression of the late apoptotic marker cleaved caspase 3. Knockdown of S100A4 resulted in a greater percentage of cells in the early and late apoptotic stages (P<0.03). Furthermore, S100A4 overexpression inhibited terminal osteogenic differentiation in both OS and MSC cell lines, whereas S100A4 knockdown stimulated terminal osteogenic differentiation. Finally, in vivo overexpression of S100A4 corresponded to significantly greater primary tumor growth and decreased doubling times (p<0.03).

DISCUSSION

We have found that S100A4 is a critical regulator of Osteosarcoma tumorigenesis. Expression of S100A4 is correlated to significantly greater OS cell proliferation, migration, and invasion in vitro. Mechanistically, S100A4 prevents cell cycle arrest and the apoptotic cascade. S100A4 also prevents terminal osteogenic differentiation in both OS cells and mesenchymal stem cells, while S100A4 knockdown promotes the differentiation cascade. Studies have shown that defective osteogenic differentiation corresponds to poorer prognoses and greater metastatic potential in OS, and prevention of this terminal differentiation by S100A4 is likely one of its mechanisms in promoting OS tumorigenesis. We believe further studies into the molecular signaling pathways regulated by S100A4 are warranted. Furthermore, it will be important to characterize the unique roles of the remaining S100 family of proteins in OS tumor progression and metastasis.

SIGNIFICANCE

We have identified a novel molecular marker that promotes OS tumorigenesis. With further investigation, we believe that targeting the S100A4 signaling pathway could be used as a treatment modality for aggressive OS.