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Growth and Metastasis

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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Malignant peripheral nerve sheath tumors (MPNSTs) are aggressive malignancies that arise within peripheral nerves. These tumors occur with increased incidence in patients with neurofibromatosis type 1 (NF1) where they exhibit increased Ras activity due to loss of the NF1 gene product, neurofibromin, and abnormal expression of the epidermal growth factor receptor (EGFR). We previously found that MPNSTs express increased levels of the CD44 family of transmembrane glycoproteins that have been implicated in tumor cell invasion and metastasis. Here we find that CD44 overexpression, driven by Src kinase activity (and not increased Ras-GTP) contributes to MPNST cell invasion. We further find that EGFR contributes at least part of the elevated Src activity in these cells. CD44 may function in concert with the c-Met receptor tyrosine kinase by promoting an autocrine loop involving the c-Met ligand, hepatocyte growth factor. The finding that inhibition of Src can effectively inhibit MPNST cell invasion warrants further study on the possible therapeutic benefits of targeting Src kinases to treat MPNST metastasis.				
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Role of CD44 in Malignant Peripheral Nerve Sheath Tumor Growth and Metastasis

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INTRODUCTION

The experiments undertaken in this study were founded on the belief that by determining molecular signals that influence malignant peripheral nerve sheath tumor (MPNST) metastasis we can gain clues about how to treat these tumors. MPNSTs are aggressive, difficult to treat malignancies that arise within peripheral nerves and infiltrate surrounding tissues (Sanguenza and Requena 1998; Ducatman et al., 1986; Sordillo et al., 1981). These tumors frequently metastasize to the lungs, lymph nodes and liver, and affected patients typically do not survive for more than five years after diagnosis (Wong et al., 1998; Ducatman et al., 1986; Ghosh et al., 1973; White et al., 1971). Although extremely rare in the general clinical population (incidence of 0.001%), recent evidence indicates that MPNSTs arise in 8-13% of patients with neurofibromatosis type I (NF1) (Evans et al., 2002) and are a major contributing factor to NF1 patient mortality (reviewed by Ferner and Gutmann et al., 2002). With the exceptions of alterations in p53, p27^{Kip1} and p16, few molecular markers have been identified which could serve as diagnostic aids or therapeutic targets for these malignancies (Kourea et al., 1999; Nielsen et al., 1999; Liapis et al., 1999; McCarron et al., 1998; Halling et al., 1996; Menon et al., 1990). We have been focusing on the role of the CD44 family of transmembrane glycoproteins in this process as we previously found that CD44 proteins are aberrantly expressed in MPNST cells and tissues (Sherman et al., 1997) and because CD44 has been implicated in the growth and metastasis of numerous human cancers (Naor et al., 1997).

BODY

In our previous progress report and preliminary studies, we reported that CD44 overexpression in MPNST cells was driven, in part, by overexpression of the epidermal growth factor receptor (EGFR) through a mechanism that depended on activation of the Src kinase. We also found that inhibition of CD44 expression in these cells or inhibition of the c-Met receptor tyrosine kinase inhibited MPNST cell invasion *in vitro*. Here, we report our results on the effects of the anti-Src kinase drug, CGP77675, on MPNST cells and show that it is an effective inhibitor of CD44 overexpression and *in vitro* invasion.

Objective 1: Determine if epidermal growth factor receptor (EGFR)-dependent Src signaling influences invasion and CD44 expression in MPNST cells

Inhibition of Src kinase activity but not MEK activity blocks MPNST cell invasion

Activating mutations in members of the Ras GTP-binding protein family have previously been implicated in tumor growth, invasion and metastasis (reviewed by Hernández-Alcoceba et al., 2000). As MPNST cells from NF1 patients have elevated levels of Ras-GTP due, at least in part, to loss of

neurofibromin, we tested whether inhibiting MEK, a downstream target of Ras-GTP, could block invasion of the ST8814 MPNST cell line derived from an NF1 patient (Glover et al., 1991). These cells have significant levels of constitutive MEK activity as measured by levels of phosphorylated ERK, which are almost completely abolished by treatment for 24 hrs. with 30 μ M of the MEK inhibitor U0126 (Fig. 1b). However, at this and higher concentrations of U0126, we observed no inhibition of MPNST cell invasion (Fig. 1a). These data indicate that the Ras-MAP kinase pathway is not required for the invasive phenotype of MPNST cells.

The activities of the Src family of non-receptor tyrosine kinases have also been implicated in tumor cell invasion (reviewed by Irby and Yeatman, 2000). Src activity is significantly higher in metastatic tissues compared to normal tissues (Talamonti et al., 1993). Furthermore, *v-src*, a constitutively activated form of *c-src*, is more potent than activated Ras in generating highly metastatic cells (Tatsuka et al., 1996). We therefore tested whether Src kinase activity contributes to MPNST cell invasion using a potent Src inhibitor, CGP77675 (Missbach et al., 1999). Compared to controls, ST8814 cells treated with CGP77675 demonstrated a dose-dependent decrease in MPNST cell invasion with nearly complete inhibition occurring at 10 μ M (Fig. 2a, b, c). Similar results were observed using a second MPNST cell line, 90-8 (data not shown). In neither case did CGP77675 influence cell proliferation as measured by a BrdU incorporation assay: 18 \pm 7% of cells treated with vehicle were positive compared to 22 \pm 9% of cells treated with CGP77675 after 24 hrs. These findings indicate that Src kinase activity contributes significantly to the invasive but not the proliferative phenotype of MPNST cells.

Src kinase but not MEK activity elevates CD44 expression in MPNSTs

Activation of both the Ras-MAP kinase pathway and Src kinase can induce CD44 transcription in different cell types (Jamal et al., 1994; Kogerman et al., 1996; Hofmann et al., 1993). Furthermore, Ras activation has been linked to the induction of alternative RNA splicing, resulting in the expression of CD44 variants that have been implicated in promoting invasion and metastasis (Weg-Remers et al., 2001). We therefore tested the effects of inhibiting Ras and Src activity on CD44 protein expression in MPNST cells. Consistent with our previous findings (Sherman et al., 1997), ST8814 cells express high levels of several CD44 splice variants, ranging from 85 kDa to 200 kDa (Fig. 1b). However, neither total CD44 levels nor the expression of CD44 variants were inhibited by the U0126 MEK inhibitor at concentrations that inhibited ERK phosphorylation (Fig. 1b). In contrast, the CGP77675 Src kinase inhibitor dramatically reduced CD44 expression in both ST8814 and 90-8 cells (60-80% compared to vehicle controls as determined by scanning densitometry; Fig. 3a). This effect was reversible, as cells treated with CGP77675 and subsequently grown in the absence of drug for 24 hrs restored high levels of CD44 expression (Fig. 3a, right panel). The effect appeared to be on the overall levels of CD44 protein expression rather than on a specific splice variant. Consistent with these results, dominant negative *c-src* transiently transfected into ST8814 cells also reduced total CD44 expression (1.7-2.1 fold compared to cells transfected with empty vector; Fig 3b). Together, these data indicate that Src but not MEK activity contributes to elevated CD44 expression in MPNST cells.

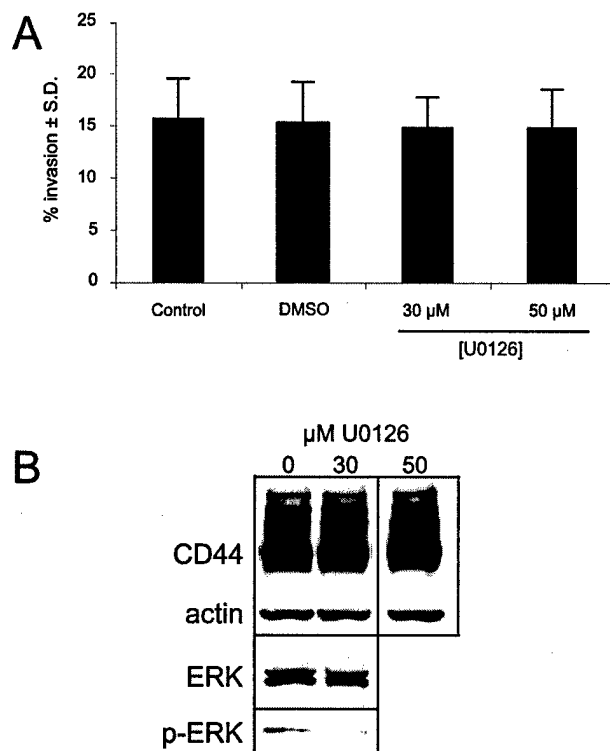


FIGURE 1: Blocking MEK activity does not influence MPNST cell invasion or CD44 expression in vitro. (A) Cells were grown on the bottom of transwells containing Matrigel and treated with different concentrations of U0126 or vehicle (DMSO). After 3 days, cells that had remained on the bottoms of the wells and those that had migrated across the transwell filters and into the Matrigel were stained with propidium iodide and examined by laser scanning confocal microscopy. Shown are the percentages of cells that invaded the Matrigel (equal to the percent of cells that reached 20 μm or deeper) at different concentrations of U0126. (B) ST8814 cells were treated with 0, 30 or 50 μM U0126 for 24 hr. then assayed for phospho-ERK and CD44 expression by western blotting. Although ERK phosphorylation was dramatically inhibited, there was no reduction in CD44 expression.

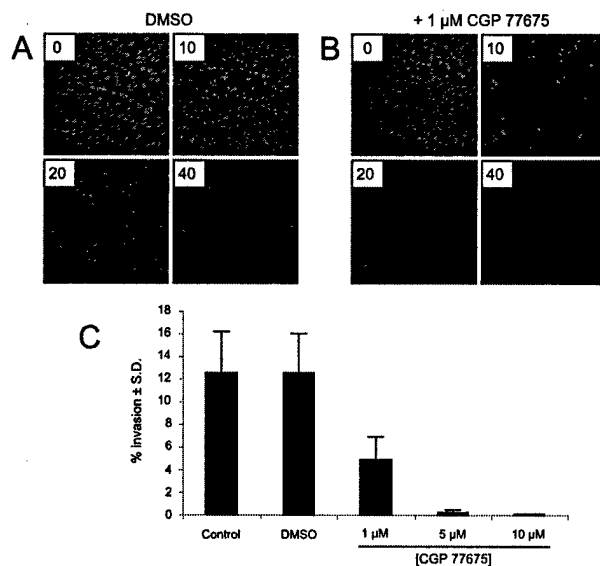


FIGURE 2: Inhibition of Src kinase inhibits MPNST cell invasion in vitro. (A, B) Confocal photomicrographs of ST8814 cells stained with propidium iodide at various distances (0-40 μm) from the bottom of the transwell filter treated with DMSO (A) or 1 μM CGP77675 (B). Note that CGP77675 inhibited both migration across the transwell filter as well as invasion into the Matrigel. (C) Quantification of the experiment in A and B, showing that CGP77675 effectively inhibited invasion. Similar results were obtained using 90-8 cells.

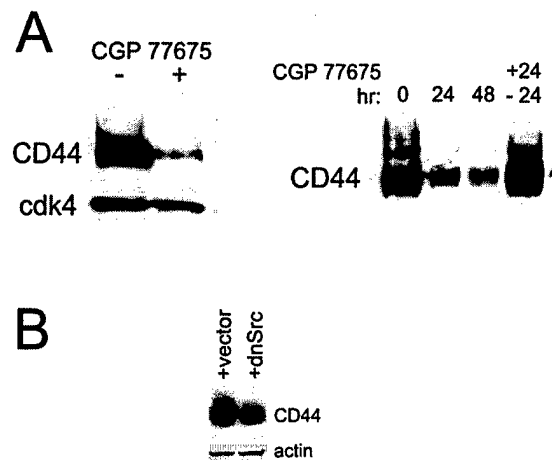


FIGURE 3: Inhibition of Src activity blocks CD44 expression by MPNST cells. (A) CD44 expression was dramatically inhibited in ST8814 cells (left panel) and 90-8 cells (right panel) treated with 5 μ M CGP77675. The effect was reversible, as cells treated for 24 hrs. in the presence of drug then grown for an additional 24 hrs. in the absence of drug ("+24/-24") restored their elevated CD44 expression levels. (B) ST8814 cells transiently transfected with dominant negative c-Src demonstrated reduced CD44 levels compared to cells transfected with empty vector.

Objective 2: Determine if CD44 contributes to MPNST cell invasion *in vitro* by influencing c-Met signaling

In our previous progress report, we reported that a c-Met ribozyme effectively inhibited c-Met expression in MPNST cells and that cells with lowered c-Met expression were significantly less invasive than cells transfected with an empty vector. We have now confirmed these results with 3 additional c-Met ribozyme-expressing MPNST clones, and each were 80-95% less invasive than corresponding control clones. We also previously found that MPNST cells utilize an HGF-c-Met autocrine loop, and that MPNST cells express a processing enzyme for HGF, HGFA. We have also confirmed that CD44 and c-Met co-immunoprecipitate with one another (data not shown) but we are awaiting the outcomes of experiments using interference-RNA (RNAi) to reduce CD44 expression and to test whether CD44 mediates c-Met activity. We have decided to use this strategy instead of the stable antisense constructs for reasons outlined below, and hope to have final results to report from these experiments within the next three months. We intend to submit a manuscript describing these results for publication.

Objective 3: Test if reducing CD44 expression inhibits MPNST growth or metastasis *in vivo*

After numerous attempts we found that we were unable to achieve the goals outlined in this aim. First, we attempted to make stable clones of MPNST cells (ST8814 line) that expressed antisense-CD44 under the control of the cytomegalovirus (CMV) promoter. We screened over 100 clones and we were able to verify, by RT-PCR, that the cells had stably integrated and were transcribing the antisense and control (scrambled antisense) constructs. However, by western blot analysis, we were unable to detect significant decreases in CD44 expression. We also attempted to generate inducible antisense clones (using the ecdysone system), but similarly found that despite transcription of the human antisense CD44 sequence, there was no appreciable reduction in CD44 protein expression.

A second problem that we encountered in control experiments was poor reproducibility with regards to tumor take rates in nude mice. In one experiment, 2 out of 10 animals developed primary tumors following subcutaneous injections of ST8814 cells into the flank. However, neither of these animals developed detectable metastatic nodules (in the lungs or other organs), and no animals developed primary tumors in two subsequent experiments. We reasoned that the lots of Matrigel we were co-injecting with the tumor cells may have contributed to this variability, but decided not to pursue the problem further in light of the difficulties with the cell clones mentioned above.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that elevated Src activity, but not Ras activity, promotes MPNST cell invasion
- Demonstrated that elevated CD44 expression promotes MPNST cell invasion
- Demonstrated that elevated Src activity, in part through aberrant expression of EGFR, leads to increased CD44 expression in MPNST cells, thus linking the effects of Src on MPNST invasion to elevated CD44
- Determined that MPNST cells utilize a c-Met-HGF autocrine loop that also promotes their invasion
- Obtained preliminary evidence that CD44 may mediate c-Met autocrine activity

REPORTABLE OUTCOMES

A manuscript entitled "Malignant Peripheral Nerve Sheath Tumor Cell Invasion is Facilitated by Src and Aberrant CD44 Expression" by Weiping Su, Mihaela Sin, Andrea Darrow and Larry Sherman has been submitted to the journal *Oncogene* and is currently under review.

An inducible antisense CD44 construct is available to other investigators who are interested in pursuing this system.

CONCLUSIONS

We conclude that CD44 overexpression, driven by Src kinase activity (and not increased Ras-GTP) contributes to MPNST cell invasion. We further find that EGFR contributes at least part of the elevated Src activity in these cells. CD44 may function in concert with c-Met by promoting an HGF-c-Met autocrine loop. The finding that an anti-Src drug can so effectively inhibit MPNST cell invasion warrants further study on the possible therapeutic benefits of targeting Src kinases to treat MPNST metastasis. As we found that blocking Src activity does not influence MPNST cell proliferation, we propose that an effective therapy for these malignancies would involve a combinatorial approach, utilizing agents that blocked both proliferation (e.g. using a farnesyl transferase inhibitor as previously described) and either an anti-Src drug or other reagents that block EGFR, CD44, or c-Met.

PERSONNEL SUPPORTED BY THIS GRANT

Larry Sherman, Ph.D. (PI) and Weiping Su, Ph.D. (post-doctoral fellow)

PUBLICATIONS

Sherman, L., Darrow, A. and Sin, M. (1999). CD44 Mediates Malignant Peripheral Nerve Sheath Tumor Cell Invasion by Facilitating c-Met Activity. *Clin. Exp. Metastasis* **17**:744

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