Published in final edited form as:

Mol Carcinog. 2010 March ; 49(3): 224–234. doi:10.1002/mc.20592.

Inhibition of Focal Adhesion Kinase and Src Increases Detachment and Apoptosis in Human Neuroblastoma Cell Lines

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Abstract

Neuroblastoma is the most common extracranial solid tumor of childhood. Focal adhesion kinase (FAK) is an intracellular kinase that is overexpressed in a number of human tumors including neuroblastoma, and regulates both cellular adhesion and survival. We have studied the effects of FAK inhibition upon neuroblastoma using adenovirus-containing FAK-CD (AdFAK-CD). Utilizing an isogenic MYCN⁺ / MYCN[−] neuroblastoma cell line, we found that the MYCN⁺ cells are more sensitive to FAK inhibition with AdFAK-CD than their MYCN negative counterparts. In addition, we have shown that phosphorylation of Src is increased in the untreated isogenic MYCN[−] neuroblastoma cells, and that the decreased sensitivity of the MYCN[−] neuroblastoma cells to FAK inhibition with AdFAK-CD is abrogated by the addition of the Src family kinase inhibitor, PP2. The results of the current study suggest that both FAK and Src play a role in protecting neuroblastoma cells from apoptosis, and that dual inhibition of these kinases may be important when designing therapeutic interventions for this tumor.

Keywords

Pediatric; Cancer; MYCN oncogene

Introduction

Neuroblastoma, the most common extracranial solid tumor of childhood, is responsible for over 15% of pediatric cancer deaths. Despite recent advances in treatments, this tumor continues to carry a dismal prognosis for children presenting with advanced or metastatic disease, with a survival of only 18–30% [1]. The primary adverse prognostic factor for neuroblastoma is amplification of the *MYCN* oncogene, which occurs in approximately a quarter of patients [2]. *MYCN* is associated with increased proliferation and cell survival, and *MYCN* knockdown results in cell death and apoptosis in neuroblastoma cell lines [3]. Unfortunately, there has been little progress made with neuroblastoma towards the development of novel therapies and improved outcomes.

Focal adhesion kinase (FAK) is a nonreceptor protein tyrosine kinase. FAK controls a number of cell signaling pathways including proliferation, viability, motility, and survival [4,5]. Phosphorylation of FAK at the tyrosine 397 (Y397) site results in a high affinity binding site

for the SH2 domain of the Src family kinases [6]. The interaction between FAK and Src results in the activation of multiple downstream pathways leading to cellular proliferation and survival. The inhibition of FAK activation has been found to affect a number of cellular pathways. FAK antisense oligonucleotides or a dominant-negative FAK protein (FAK-CD) has been shown to cause apoptosis and decreased growth in human breast cancer cells and melanoma cells [7,8,9]. Silencing FAK expression with small interfering RNAs resulted in decreased migration of lung cancer [10] and glioblastoma cells [11], and increased apoptosis in ovarian cancer cells [12]. Initial studies from our laboratory have revealed that both the abundance of FAK mRNA and the expression of FAK protein are significantly increased in human neuroblastomas [13]. In addition, we recently demonstrated that *MYCN* regulates the expression of FAK through its promoter in human neuroblastoma cell lines and that *MYCN*+ cell lines have increased FAK expression [14].

Src is another nonreceptor protein tyrosine kinase that is overexpressed in a variety of human tumors including breast and colon cancer and neuroblastoma [15,16,17,18]. Src has been shown to have a role in neuroblastoma cell survival [19]. Previously, we showed that inhibition of both FAK and Src resulted in an increase in apoptosis in colon cancer cells [20].

Since FAK is overexpressed in *MYCN*+ neuroblastoma cell lines, we hypothesized that inhibition of FAK may result in decreased cell viability and apoptosis in these cells. In the current studies, we show that *MYCN* expressing neuroblastoma cells are more sensitive to FAK inhibition by Ad-FAK-CD than the isogenic *MYCN* non-expressing neuroblastoma cell line. In addition, we show that the effects of Src inhibition along with FAK inhibition in neuroblastoma cell lines is additive, and therefore dual inhibition of FAK and Src may be necessary when designing therapeutic interventions for neuroblastoma.

Materials and Methods

Cells and Cell Culture

The Tet-off *MYCN* +/- cell line, (Tet-21/N or SHEP-21/N), was generously provided by Dr. S. L. Cohn (Northwestern University's Feinberg School of Medicine, Chicago, IL) with permission from Dr. M. Schwab (Deutsches Krebsforschungszentrum, Heidelberg, Germany) [21]. These cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 µg/mL penicillin and 1 µg/mL streptomycin, and grown in the presence or absence of tetracycline (1 µg/mL) for 48–72 hours for *MYCN*– (Tet+) and *MYCN*+ (Tet–) cells, respectively.

Adenoviral Infections

Cells were plated in equal numbers and allowed to attach for 24 hours. The cells were then infected with control AdLacZ or AdFAK-CD [7]. The AdFAK-CD construct is an adenoviral construct that contains the carboxy-terminal domain of FAK (FAK-CD). The COOH-terminal domain of FAK is analogous to FAK-related non-kinase (FRNK), known to decrease the phosphorylation of p125^{FAK}. As such, AdFAK-CD functions as a dominant-negative for FAK. AdFAK-CD has been previously described in detail by Xu *et al* [7]. Optimal concentrations of virus were determined as described previously [7,14]. We used viral titers that caused expression of AdLacZ as checked by X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside) staining in > 90% of the cells without any toxic effect, and was equal to 500 ffu per cell, as previously described [9,20].

Antibodies and Reagents

Monoclonal anti-FAK (4.47) and polyclonal anti-phospho-FAK (Y397) and anti-*MYCN* (9405) antibodies were obtained from Upstate Biotechnology, Inc. (Upstate, NY), and

Biosource (Invitrogen, Carlsbad, CA), respectively. Monoclonal antibodies for total poly (ADP-ribose) polymerase (PARP, 611038) were obtained from BD Transduction Labs (BD PharMingen, San Jose, CA), for caspase 9 (9502), cleaved PARP (9532), AKT (9272), phospho-AKT (Ser 473, 9271S), ERK (9102) and phospho-ERK (43775) from Cell Signaling Technology, Inc. (Danvers, MA), and for phospho-Src (44–660G) from Biosource (Invitrogen). Monoclonal antibodies against Src (SC-18) and β -actin were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine), an inhibitor of Src phosphorylation, was obtained from Calbiochem (San Diego, CA) and used at a concentration of 30 μ M [30].

Western Blotting

Western blots were performed as previously described [14]. Briefly, antibodies were used according to manufacturer's recommended conditions. Molecular weight markers were used to confirm the expected size of the target proteins. Immunoblots were developed with chemiluminescence Renaissance Reagent (PerkinElmer Life Sciences, Waltham, MA). Blots were stripped with stripping solution (Bio-Rad, Hercules, CA) at 37 °C for 15 minutes and then reprobed with selected antibodies. Immunoblotting with antibody to β -actin provided an internal control for equal protein loading. Densitometry evaluation of immunoblots was performed utilizing Scion Image for Windows (National Institutes of Health).

Detachment Assay

Equal numbers of cells were plated and allowed to attach for 24 hours. The cells were then treated with nothing, AdLacZ, or AdFAK-CD for 24 or 48 hours with or without PP2. Detached and adherent cells were collected separately and counted with a hemacytometer. The percentage of detached cells was calculated by dividing the number of detached cells by the total number of cells (detached plus adherent), and expressed as a percentage.

Cell Viability Assays

Equal numbers of MYCN⁺ and MYCN[−] cells were plated and allowed to attach for 24 hours. The cells were treated with nothing, AdLacZ, or AdFAK-CD for 24 or 48 hours with or without PP2 and cellular viability was measured using trypan blue exclusion and cell counting with a hemacytometer. Viability was further measured with Cell Titre 96 AQueous One solution assay kit (Promega, Madison, WI). In brief, cells were plated 5×10^3 cells per well on 96-well culture plates and allowed to attach. Following 48 hours of treatment, 20 μ L of Cell Titer 96 AQueous One solution reagent was added to 100 μ L of cell medium. After 1–4 h, the absorbance at 490 nm was measured using a kinetic microplate reader ($V_{\max}$, Molecular Devices).

Cell proliferation assay

Cellular proliferation was measured using BrdU incorporation assay. This immunoassay quantitates the incorporation of bromodeoxyuridine into newly synthesized DNA, thereby providing a mechanism to measure cellular proliferation. A BrdU Cell Proliferation Assay (Calbiochem, San Diego, CA) was used according to manufacturer's instructions. Briefly, 2.5×10^3 cells were plated onto 96 well plates. Cells were treated with the AdLacZ or AdFAK-CD for 48 hours. BrdU was added and after 2 hours the cells were collected and fixed. Anti-BrdU antibody was added followed by horseradish peroxidase-conjugated secondary antibody. A fluorometer is utilized to detect the amount of BrdU incorporated into the cells.

Apoptosis Assays

Apoptosis was determined by three methods. Following treatment with nothing, AdLacZ, or AdFAK-CD for 24, or 48 hours, with or without PP2, cells were stained with Hoechst 33258 as previously described [20,22]. Cells undergoing apoptosis have condensation and

fragmentation of nuclei. Hoechst stain binds to DNA and demonstrates condensed chromatin or micronuclei in cells that are undergoing apoptosis. The cells (both floating and adherent) are harvested, fixed to a glass slide, stained with Hoechst 33258, positive cells counted with fluorescence microscopy, and a percentage of apoptotic cells calculated in three independent fields with 100 nuclei per field.

Apoptosis was also detected by immunoblotting for PARP expression. During apoptosis, poly (ADP-ribose) polymerase (PARP) is cleaved. The disappearance of the total protein or the accumulation of cleaved protein as detected by immunoblotting are methods that can be utilized to detect apoptosis. Cells are treated as described, lysates are collected, and immunoblotting is performed. Bands are detected by chemiluminescence and β -actin serves as an internal control.

Flow cytometry was also used to detect apoptosis. DNA fragmentation in apoptotic cells can be detected utilizing TdT end-labeling (TUNEL). The APO-BRDU™ kit (BD PharMingen) is a two color staining method for labeling DNA strand breaks that can then be detected by flow cytometry. The treated cells (both adherent and floating) are harvested and labeled according to manufacturer's instructions. For each of the studies, specimens are stained according to manufacturer's instructions and analyzed with a FACSCalibur™ machine gated to exclude cellular debris and evaluating 10^5 events. Calculations were completed with Cellquest™ Software (Becton Dickinson Systems, San Jose, CA). All methods of apoptosis detection gave similar results. In addition, previous work from our laboratory has shown that TUNEL and Hoechst staining produce the same data (9).

Data Analysis

Experiments were repeated at least in triplicate, and data are reported as mean $\pm$ standard error of the mean (SEM). An ANOVA or student's t-test was used as appropriate to compare data between groups. Statistical significance was determined at the $P < 0.05$ level.

Results

AdFAK-CD treatment causes cellular rounding and detachment

We have previously shown that FAK is associated with *MYCN* expression in human neuroblastomas [13,14]. In addition, previous data utilizing a dominant-negative FAK construct or FAK siRNA showed that *MYCN* expressing neuroblastoma cell lines may be more dependent upon FAK for survival than non-*MYCN* expressing neuroblastoma cell lines [14]. Therefore, we wished to define the biologic significance of interruption of FAK function in human neuroblastoma cells. We utilized an isogenic neuroblastoma cell line that has a tetracycline repressible *MYCN* expression vector; that is, when tetracycline is present, *MYCN* is silenced (*MYCN*⁻, Tet⁺), and *MYCN* is expressed when tetracycline is removed from the media (*MYCN*⁺, Tet⁻). To inhibit FAK, we utilized a dominant-negative construct for FAK, AdFAK-CD which has been previously described in detail [7]. Optimal concentrations of virus were determined as previously described [7,14]. AdLacZ was used as a control at the same multiplicity of infection. These conditions resulted in effective infectivity of the neuroblastoma cell lines as assessed by X-gal staining for AdLacZ. Both the *MYCN*⁺ and *MYCN*⁻ neuroblastoma cells infected with AdLacZ expressed β -galactosidase, as detected by X-gal staining (data not shown). Using phase contrast microscopy, we demonstrated that the effect of AdFAK-CD upon neuroblastoma cellular morphology was related to the *MYCN* status of the cells (Fig. 1A). *MYCN*⁺ (Tet⁻) and *MYCN*⁻ (Tet⁺) cells were plated in equal numbers. After 48 hours of AdFAK-CD treatment, there was an obvious change in cell morphology consisting of cell rounding and floating most marked in the *MYCN*⁺ (Tet⁻) cells (Fig. 1A,

bottom right panel). Control AdLacZ had no effect upon the morphology of either cell line (Fig. 1A, *middle panels*).

Since FAK is known to play an important role in cellular attachment and many of the cells were noted to be floating, we examined the effects of AdFAK-CD upon cellular detachment. We compared the MYCN⁻ (Tet⁺) and the MYCN⁺ (Tet⁻) neuroblastoma cell lines and measured the percentage of cells that were detached after AdFAK-CD treatment. The number of detached cells was determined as follows: floating and adherent cells were collected separately and counted. The percentage of detached cells was calculated by dividing the number of floating cells by the total number of cells collected (floating plus adherent). For these experiments, MYCN⁺ (Tet⁻) and MYCN⁻ (Tet⁺) cells were plated in equal numbers and allowed to attach for 24 hours. After 48 hours of AdFAK-CD treatment, there was significantly more detachment in the MYCN⁺ cells than in the MYCN⁻ cells (Fig. 1B). Treatment of these two cell lines with control AdLacZ did not have a significant effect upon detachment (Fig. 1B). Thus, treatment with AdFAK-CD leads to increased neuroblastoma cellular rounding and detachment which was most marked in the MYCN⁺ cell line.

Inhibition of FAK with AdFAK-CD leads to decreased cellular proliferation and viability and increased cellular apoptosis

Since FAK has an important role in cellular survival, we wished to determine the effects of FAK inhibition upon proliferation, viability, and apoptosis in neuroblastoma. Cellular proliferation in the MYCN⁺ and MYCN⁻ cell lines was measured with BrdU incorporation assays. After 48 hours of AdFAK-CD treatment, there is a significant decrease in cellular proliferation in both cell lines, but the proportional amount of decrease is significantly greater for the MYCN⁺ cells than for the MYCN⁻ cells (Fig. 2A). In addition, we measured cell viability with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assays and found that after 48 hour of AdFAK-CD treatment, there is a significant decrease in viability in both cell lines compared to non-treated controls, but again, the decrease is more pronounced in the MYCN⁺ cell line (Fig. 2B). These results are similar to those seen with proliferation.

We next examined the effects of AdFAK-CD upon neuroblastoma cellular apoptosis in these isogenic cell lines. Using TUNEL staining and FACS analysis, we found that AdFAK-CD treatment for 48 hours leads to significant increases in apoptosis in both the MYCN⁺ and MYCN⁻ cells, but as noted with the cell proliferation and viability experiments, the difference is more marked in the MYCN⁺ than the MYCN⁻ cells (Fig. 2C). Biochemical confirmation of apoptosis was demonstrated by immunoblotting for cleaved PARP (Fig. 2D). There is an increase in expression of cleaved PARP detected in the MYCN⁺ cells treated with AdFAK-CD compared to the AdFAK-CD treated MYCN⁻ cells, consistent with the TUNEL results. AdLacZ had no significant effect on cell proliferation, viability, or apoptosis. These data clearly show that FAK inhibition with AdFAK-CD has significant biologic effects upon neuroblastoma cellular viability and survival that is more marked in the MYCN⁺ cells.

AdFAK-CD causes dephosphorylation of FAK in MYCN⁺/– neuroblastoma cells

Next, to determine a mechanism for the biologic effects of AdFAK-CD upon neuroblastoma cells, we compared the effects of AdFAK-CD upon FAK expression in the MYCN⁺ and MYCN⁻ cell lines. After 48 hours of treatment with AdFAK-CD, there is a decrease in phosphorylated FAK (Y397) in both cell lines (Fig. 3A), that is confirmed with densitometry evaluation of the immunoblot (Fig. 3B). AdLacZ treatment did not affect phosphorylated or total FAK expression (Fig. 3A, B). We also probed our lysates with antibodies to other tyrosine phosphorylation sites on FAK known to interact with Src such as FAK Y925, FAKY891, FAKY407, FAKY576 and FAKY577. None of these sites, in either the MYCN⁺ or MYCN⁻ cells were affected by AdFAK-CD treatment (data not shown). Therefore, AdFAK-CD leads

to FAK dephosphorylation at the Y397 autophosphorylation site. This loss of FAK function through dephosphorylation explains the decreased cell attachment and survival which is seen more prominently in the MYCN+ cells, implying that they are more dependent upon FAK as a survival mechanism than the MYCN- cells.

Src phosphorylation is increased in MYCN- cell line

To further investigate the less vigorous response of the MYCN- neuroblastoma cells to AdFAK-CD-induced dephosphorylation of FAK, we examined the expression of other protein kinases that are related to FAK and have been shown to be important for neuroblastoma proliferation and survival such as Src [16,18], Erk [23,24], and Akt [25,26]. We found a difference in phospho-Src, with the MYCN- cells showing more phospho-Src than the MYCN+ cells (Fig. 3C), confirmed with densitometry evaluation (Fig. 3D). However, we found no differences in the expression of phospho- or total Erk or in phospho- or total Akt expression between the two cell lines (Fig. 3C, rows 4, 5, 6, 7). This increased level of Src phosphorylation in the MYCN- cells suggested that Src may play a role in the diminished response seen in the MYCN- neuroblastoma cells to the down-regulation of FAK with AdFAK-CD.

Dual inhibition of FAK and Src leads to increased detachment, decreased viability and increased apoptosis

Because we have previously shown that dual inhibition of FAK and Src results in increased apoptosis in colon cancer cells [20] and since we now noted differences in phospho-Src between the MYCN+ and MYCN- cells, we chose to investigate the dual effects of Src and FAK inhibition upon these isogenic neuroblastoma cells. We used Src-family kinase inhibitor, PP2, to inhibit Src in these two isogenic neuroblastoma cell lines. Phosphorylated Src expression was successfully inhibited following treatment with PP2 (Fig. 4A, B). In addition, in these two cell lines, PP2 treatment had no effect upon other kinases such as Erk and Akt (Fig. 4A, rows 3, 4, 5, 6). There was a decrease in Y397 phospho-FAK in both cell lines with PP2 treatment (Fig. 4C, D).

We first tested the effects of FAK down-regulation with and without inhibition of Src on cellular detachment and viability in MYCN+ and MYCN- isogenic neuroblastoma cell lines. We found minimal effects of the control AdLacZ or AdLacZ with PP2 on detachment (Fig. 5A), indicating that the decrease in phospho-FAK with PP2, did not appear to have biologic significance. However, as early as 24 hours after treatment with dual inhibition with both AdFAK-CD and PP2, there was a significant increase in detachment of the MYCN+ and MYCN- cells, with detachment increased in the MYCN- cells over 2.5 times that of AdFAK-CD alone and over 1.5 times in the MYCN+ cells (Fig. 5A), demonstrating that dual inhibition of FAK and Src increased cellular detachment. In addition, we measured cell viability with trypan blue staining (Fig. 5B). Non-viable cells were counted and expressed as a fold change, comparing treated to non-treated control cells. As with detachment, there was a significant increase in the change in non-viable cells as early as 24 hours after dual inhibition with both AdFAK-CD and PP2, in the MYCN+ and MYCN- cells, with non-viability increased in the MYCN- cells over 3 times that of AdFAK-CD alone, demonstrating that dual inhibition of FAK and Src decreased cellular viability.

Next, we evaluated apoptosis using Hoechst staining. We found that dual inhibition of FAK and Src with AdFAK-CD and PP2, respectively, resulted in a significant increase in apoptosis. There was little effect upon apoptosis by AdLacZ alone or with PP2 treatment (Fig. 5C). However, in as early as 24 hours after dual inhibition, both cell lines showed significant increase in apoptosis, with an increase over 3 times that seen with AdFAK-CD alone (Fig. 5C). To further characterize apoptosis biochemically, immunoblotting was performed for total PARP and caspase 9. After 24 hours, treatment with AdFAK-CD leads to a loss of expression of total

PARP and total caspase 9 in both cell lines, which becomes even more marked in the MYCN⁻ cells after the addition of PP2 to the AdFAK-CD treatment (Fig. 5D, rows 1, 2, 3). Treatment with AdLacZ with or without PP2 did not affect the cells (Fig. 5D). These data clearly show that inhibition of FAK and Src together results in an increased effect upon apoptosis.

Discussion

Focal adhesion kinase (FAK) has been shown to be overexpressed in a number of human tumors [27,28] including colon [29], breast [29], ovarian [30], and thyroid cancers [31], and is thought to be a key factor in tumorigenesis. The inhibition of FAK has been shown to lead to loss of adhesion and apoptosis in tumor cells. Since we have recently shown that FAK is overexpressed in MYCN expressing neuroblastoma cell lines [13,14], we hypothesized that FAK is important in cell survival in MYCN amplified neuroblastomas. To evaluate that hypothesis, we undertook the current study using an isogenic MYCN^{+/-} neuroblastoma cell line and have clearly shown that FAK inhibition results in increased detachment and apoptosis in neuroblastoma cells. The effects of AdFAK-CD-induced FAK inhibition appear to be more pronounced in the cells that are MYCN⁺, suggesting that these cells have a greater dependence upon FAK for survival than their MYCN⁻ counterparts.

Our current data show that FAK inhibition with AdFAK-CD in neuroblastoma cells results in a loss of FAK (Y397) phosphorylation and decreased neuroblastoma cell survival. Tyr-397 is the major autophosphorylation site of FAK [6,32], and activation at this site results in the binding and activation of other signaling molecules that ultimately result in decreased apoptosis [33]. Other authors have shown that other methods of FAK Tyr-397 dephosphorylation result in cellular apoptosis and decreased tumor survival. For example, treatment with NVTAE-226, a FAK kinase inhibitor, resulted in FAK (Y397) dephosphorylation and apoptosis in breast cancer cells [34], and in an ovarian carcinoma model [35]. Interestingly, in 2003 Kim and colleagues reported that treatment of SH-EP neuroblastoma cells with okadaic acid resulted in loss of FAK phosphorylation at Tyr397 and resulted in apoptosis in this cell line [36].

We noted in this study that Src was expressed by both of the isogenic cell lines, but that phosphorylated Src was greater in the MYCN⁻ cells. Other investigators have noted similar findings when studying Src in neuroblastoma. Bjelfman and colleagues showed that Src phosphorylation increased in SH-SY5Y, a MYCN non-amplified neuroblastoma cell line [37]. Other investigators have studied the expression of Src in human neuroblastomas in relation to the MYCN oncogene. Matsunaga *et al* evaluated neuroblastoma cell lines and infantile neuroblastoma tumor specimens and found an inverse correlation between *src* mRNA levels and amplification of the MYCN oncogene [18,38]. Others have noted that *src* proto-oncogene signal transduction was related to neuronal differentiation and therefore, prognosis in neuroblastoma tumors [17]. The above studies concluded that since MYCN is normally only expressed in undifferentiated neuronal cells, its expression in neuroblastoma arises from immature cells, thereby enhancing the maintenance of the malignant phenotype of the tumor. Since *src* expression and kinase activity is known to increase with neural differentiation, it follows that an inverse relationship would exist between these two entities. Although MYCN generally functions as a transcription factor, no direct relationship between MYCN and *src* has yet been described.

We have previously shown that MYCN upregulates FAK expression [14]. FAK and Src activation usually correlate directly, but in our study, we found an inverse correlation between FAK and Src in these isogenic MYCN cell lines. There are other reports of inverse correlation between FAK and Src. Sieg *et al* found an upregulation in Src family kinases in cell lines that have diminished FAK function [39]. These investigators found that the protein tyrosine kinase activity of Src was significantly increased in FAK^{-/-} cells when compared to FAK^{+/+} cells,

and felt that this increase in kinase activity may be responsible for a compensatory signaling pathway in FAK^{-/-} cells. In addition, other authors have found that Src has cellular survival functions that are independent of FAK. Moissoglu and Gelman [40] utilized FAK^{-/-} mouse embryonic fibroblasts transformed with v-Src to study the FAK independent oncogenic potential of Src. They found a significant increase in soft agar colony formation and migration in the v-Src transformed cells, both of which were independent of FAK function. Specifically in neuroblastoma, Finlay and Vuori demonstrated that Src was activated after caspase 8 was reconstituted in neuroblastoma cells. This Src activation was shown to be independent from FAK activation, and it promoted cell adhesion and activation of other downstream survival pathways [19]. So although we have shown that FAK function is decreased in MYCN⁻ cells compared to their MYCN⁺ counterparts, Src function may still be increased in these cells, and may be providing a survival advantage that is relatively independent of FAK. Therefore, decreasing both pathways would result in findings similar to those in our study with Src inhibition decreasing cell survival in those cells that are both FAK-insensitive and further decreasing survival in those that are FAK-sensitive.

It is interesting that although both the MYCN⁺ and MYCN⁻ cell lines expressed Src, inhibition with the Src-family kinase inhibitor, PP2, alone did not result in significant apoptosis in either cell line. Hiwasa and others, utilizing a neuroblastoma cell clone that lacked Src-phosphorylation, showed that Src activity, specifically phosphorylation, was necessary to prevent SH-SY5Y neuroblastoma cells from undergoing herbimycin A-induced apoptosis. Their findings indicated that another event in addition to Src inhibition was required for cellular apoptosis [41]. Other authors have noted that Src specific inhibitors capable of Src dephosphorylation do not result in decreased cell survival in all tumor cell types [42]. In addition, it has been shown that silencing Src with siRNA also results in decreased Src activation without an increase in apoptosis in human pancreatic cancer cells [43]. Src inhibition has been shown to increase chemosensitivity of many human tumor cell lines, and Ischenko and colleagues speculated that Src may regulate the response of cancer cells to chemotherapy by modulating mitochondrial apoptosis pathways [43]. In fact, Karni et al demonstrated that Src regulates the expression of Bcl-2 proteins [44]. Therefore, it may be a useful therapeutic strategy to combine Src inhibition with other therapies including the inhibition of other survival signaling kinases such as FAK.

In the current study, we found that the effects of dual inhibition of FAK and Src in the MYCN⁻ cell line was so marked, that differences were noted in our assays as early as 24 hours after dual treatment that were not relevant in the MYCN⁻ cell line even after 48 hours of treatment with AdFAK-CD alone. The dual targeting of these two kinases has been shown in the past to be an effective *in vitro* strategy against colon [20,46] and breast cancer cells [47]. In addition, in a recent study evaluating the FAK-mediated Src activation in human neuroblastomas, Wu and colleagues [48] suggested that neuroblastoma tumor progression may require FAK and Src. Our dual inhibition data indicate that targeting Src in addition to FAK results in an increase in apoptosis in both the MYCN⁺ and MYCN⁻ cells lines.

Phosphorylation at the Tyr-397 site on FAK provides a high affinity binding site for the SH2 domain of the Src family kinases that results in decreased apoptosis through the induction of inhibitor of apoptosis proteins and inhibition of members of the pro-apoptotic Bcl-2 family [45]. Src-mediated apoptosis is also linked to p53 upregulation and induction of the pro-apoptotic protein Bax [49], leading one to believe that p53 status of neuroblastoma may be important to our findings. Previous authors, using the same isogenic MYCN⁺/MYCN⁻ cell line, have shown that MYCN cooperated with cytotoxic drugs to increase p53 and Bax protein expression leading to increased apoptosis [50]. Since MYCN amplification is associated with poor prognosis in neuroblastoma, and neuroblastoma tumors are almost uniformly p53 wild-type, we must hypothesize, as these authors did, that dysfunctions in other apoptotic pathways

must exist as a mechanism that neuroblastomas employ for survival. Chen et al recently showed that MDM2, a primary inhibitor of p53, is a direct transcriptional target of MYCN, and that MDM2 knockdown in p53 wild-type neuroblastoma tumor xenografts resulted in decreased tumor growth [51]. Golubovskaya showed that wild-type p53 binds to the FAK promoter and inhibits FAK protein expression [52], but we have demonstrated that MYCN binds to the FAK promoter to increase FAK protein expression [14]. Therefore, although p53 wild-type is the norm in neuroblastomas, its apoptotic effects are likely abrogated by other mechanisms involving transcriptional regulation through MYCN.

One critique of the methods utilized in our study may be the use of AdFAK-CD to inhibit FAK phosphorylation. We chose this method, since it is well known to our laboratory, and the FAK kinase inhibitors currently available, that is NVTA-226, is not entirely specific for FAK kinase activity [53]. In addition, the choice of PP2 as a Src inhibitor must also be discussed, since this compound may also interfere with other kinases [54]. In our cell lines, PP2 inhibited Src phosphorylation without interfering with phosphorylation of Akt or Erk. The differences seen in FAK phosphorylation were present in both cell lines, but did not appear to have a significant biologic effect as noted by no changes in detachment, viability or apoptosis in the cell lines investigated. Therefore, despite these two points, we believe that these data provide a fundamental framework for further studies with this pathway in neuroblastoma.

The molecular basis for the aggressive behavior of neuroblastoma has not been elucidated. An important finding in this study is that the relative resistance to FAK inhibition with AdFAK-CD in MYCN⁻ neuroblastoma cells is abrogated by the addition of PP2. PP2 was also able to increase AdFAK-CD induced apoptosis in the MYCN⁺ neuroblastoma cell line. Since 80% of neuroblastomas are MYCN⁻, but the MYCN⁺ tumors are very aggressive, combining these two therapies may be an effective therapeutic strategy for both MYCN⁻ and MYCN⁺ neuroblastomas. Our study provides data in neuroblastoma that indicate that FAK-independent Src-mediated survival functions in neuroblastoma will be important to explore in the future.

Acknowledgments

We thank Dr. S. L. Cohn and Dr. M. Schwab for their kind gift of the MYCN⁺/MYCN⁻ cells (N-myc⁺/-, Tet-21/N or SHEP-21/N). The project described was supported by a Grant Number K08CA118178 from the National Cancer Institute (EAB). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Cancer Institute or the National Institutes of Health.

Abbreviations

FAK	focal adhesion kinase
PP2	4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
ffu	focus forming units
AdFAK-CD	adenoviral focal adhesion kinase C-terminal domain
PARP	poly (ADP-ribose) polymerase
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

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Fig. 1A

Fig. 1B

Fig. 1.

FAK inhibition with AdFAK-CD increases cellular rounding and detachment in MYCN+ neuroblastoma cells. **A**, Representative images from phase microscopy. Equal numbers of MYCN+ and MYCN- neuroblastoma cells were plated and allowed to attach. These cells were then treated for 48 hours with nothing, AdFAK-CD or AdLacZ. There is a significant decrease in the number of cells and an increase in cellular rounding after AdFAK-CD treatment in the MYCN+ cells after 48 hours (*bottom right panel*). The MYCN- cells show less cell loss and rounding in response to AdFAK-CD treatment, but there are changes in cell morphology present at 48 hours (*bottom left panel*). **B**, Equal numbers of MYCN+ and MYCN- cells are plated and allowed to attach. The cells are then treated with nothing, AdFAK-CD, or AdLacZ for 48 hours. Floating and adherent cells are collected and counted. The percentage of detached cells is calculated by dividing the number of floating cells by the number of total cells (floating plus adherent cells). After 48 hour of treatment, the percentage of detachment is significantly increased in the AdFAK-CD treated MYCN+ neuroblastoma tumor cells. Treatment with AdFAK-CD has little effect upon detachment in the MYCN- cell line. AdLacZ does not significantly affect detachment in either cell line.

Fig. 2A

Fig. 2B

Fig. 2C

Fig. 2D

Fig. 2.

FAK inhibition with AdFAK-CD results in decreased proliferation and viability and increased apoptosis in neuroblastoma cell lines. Equal numbers of MYCN+ and MYCN- cells are plated and allowed to attach. MYCN+ and MYCN- neuroblastoma cells were then treated for 48

hours with nothing, AdLacZ, or AdFAK-CD. Experiments are repeated at least three times and data reported as mean $\pm$ SEM. **A**, Proliferation is measured with BrdU assay (Calbiochem) according to manufacture's instructions. Data are reported as fold change in proliferation compared to untreated control cells. After treatment with AdFAK-CD, there is a significant decrease in proliferation in both cell lines, but the decrease is greater in the MYCN+ cells. Treatment with the control, AdLacZ, does not significantly affect proliferation in either cell line. **B**, Viability is measured with MTT assay. Data are reported as fold change in viability compared to untreated control cells. Findings are similar to the proliferation results, with significant decreases in viability after AdFAK-CD treatment in both the MYCN+ and MYCN- cell lines, but the change is more marked in the MYCN+ cells. Again, treatment with AdLacZ control does not significantly affect viability in either cell line. **C**, Apoptosis is measured with TUNEL staining by FACS analysis. The APO-BRDU™ kit (BD PharMingen) is a two color staining method for labeling DNA strand breaks that can then be detected by flow cytometry. After 48 hours, the treated cells are harvested and labeled according to manufacturer's instructions and analyzed with a FACSCalibur™ machine gated to exclude cellular debris and evaluating 10⁵ events. AdFAK-CD treatment leads to significant increases in apoptosis in both the MYCN+ and MYCN- cells, but as noted with the cell proliferation and viability experiments, the difference is more marked in the MYCN+ cells. **D**, Apoptosis is confirmed biochemically by detection of cleaved PARP products by Western blot. After 48 hours of treatment, the cells were harvested and cell lysates collected. Western blot was performed as described in the Methods section, utilizing an antibody that detects PARP cleavage products. There is a significant increase in cleaved PARP detected after treatment with AdFAK-CD, but again, the increase is more marked in the MYCN+ neuroblastoma cell line compared to that of the MYCN- cells.

Fig. 3A

Fig. 3B

Fig. 3C

Fig. 3D

Fig. 3.

AdFAK-CD treatment results in decreased phosphorylation of FAK (Y397). **A**, Equal numbers of MYCN+ and MYCN- cells were plated and treated with nothing, AdLacZ or AdFAK-CD for 48 hours. Cell lysates were collected and proteins separated by SDS-PAGE. Lysates were probed with anti-phosphotyrosine FAK antibodies including Tyr-407, Tyr-576, Tyr-577, Tyr-861, Tyr-925 (data not shown) and the autophosphorylation site Tyr-397. Representative immunoblot shows decreased Tyr-397 FAK expression after treatment with AdFAK-CD for 48 hours in both cell lines. To control for equal protein loading Western blotting was performed with β -actin antibody. **B**, The immunoblot results are corroborated with densitometry evaluation for Tyr-397 FAK. Relative Tyr-397 FAK is determined by comparing the density of the Tyr-397 FAK band to the density of the total FAK band for each treatment group. Data are reported as mean $\pm$ sem. **C**, The expression of other protein kinases related to FAK was investigated in the isogenic MYCN+ and MYCN- cell lines. Western blot (Materials and Methods Section) was performed with Src, phospho-Src, Erk, phospho-Erk, Akt, and phospho-Akt antibodies. There was no difference in expression of Erk or Akt proteins or in their phosphorylation between the MYCN+ and MYCN- cell lines. Phospho-Src is increased in the MYCN- cell lysates compared to the lysates of the isogenic MYCN+ cell line. Western blotting was performed with β -actin antibody to verify equal protein loading. **D**, Relative phospho-Src is determined with densitometry evaluation of immunoblot by comparing the density of the phospho-Src band to the density of the total Src band for each treatment group. Data are reported as mean $\pm$ sem. Densitometry corroborates increased phospho-Src seen in MYCN- versus MYCN+ cells.

Fig. 4A

Fig. 4B

Fig. 4C

Fig. 4D

Fig. 4.

Inhibition of Src with PP2 leads to decreased phosphorylation of Src. **A**, MYCN+ and MYCN- cells were treated with PP2 for 24 hours. Western blot (Materials and Methods Section) was performed with Src, phospho-Src, Erk, phospho-Erk, Akt, and phospho-Akt antibodies. There was no effect in either cell line upon the expression of Erk or Akt proteins or in their phosphorylation after PP2 treatment. However, PP2 treatment results in decreased Src phosphorylation in both the MYCN+ and MYCN- cell lysates. Western blotting was performed with β -actin antibody to verify equal protein loading. **B**, Relative phospho-Src is determined with densitometry evaluation of immunoblot. The density of the phospho-Src band is expressed as a ratio to the density of the total Src band for each treatment group. Data are reported as mean $\pm$ sem. Densitometry corroborates decreased phospho-Src seen in MYCN+ and MYCN- cell lines after PP2 treatment. **C**, MYCN+ and MYCN- cells were treated with PP2 for 24 hours and Western blot (Materials and Methods Section) was performed with FAK and Tyr-397 FAK antibodies. PP2 treatment results in decreased phosphorylation of Tyr-397 FAK in both the MYCN+ and MYCN- cell lines. Western blotting was performed with β -actin antibody to verify equal protein loading. **D**, Relative phospho-FAK (Y397) is determined with densitometry evaluation of immunoblot. The density of the phospho-FAK band is expressed

as a ratio to the density of the total FAK band for each treatment group. Data are reported as mean $\pm$ SEM. Densitometry shows decreased phospho-FAK seen in MYCN⁺ and MYCN⁻ cell lines after PP2 treatment.

Fig. 5A

Fig. 5B

Fig. 5C

Fig. 5D

Fig. 5.

Dual inhibition of FAK and Src leads to increased detachment, decreased viability and increased apoptosis. Equal numbers of MYCN+ and MYCN- neuroblastoma cells were plated and then treated for 24 hours with nothing, AdLacZ, or AdFAK-CD, with or without the addition of PP2. Experiments are repeated at least three times and data reported as mean $\pm$ SEM. **A**, After treatment, floating and adherent cells are collected separately and counted. The percentage of detached cells is calculated by dividing the number of floating cells by the number of total cells (floating plus adherent cells). After just 24 hours of dual treatment with AdFAK-CD and PP2, significant detachment is seen in both cell lines. **B**, Cell viability was determined with trypan blue staining. After 24 hours of dual inhibition with AdFAK-CD and PP2, all cells were collected, stained with trypan blue, and counted. Experiments were repeated at least three times, and data reported as fold change in viability. There was a significant increase in the non-viable cells in the MYCN+ and MYCN- cells as early as 24 hours after dual inhibition with AdFAK-CD and PP2, with non-viability increasing in the MYCN- cells over 3 times that of AdFAK-CD alone. **C**, After 24 hours treatment with dual inhibition using AdFAK-CD and PP2, cells were collected, fixed, stained with Hoechst, and counted. Apoptosis was measured by counting the number of stained cells. As early as 24 hours after initiating dual inhibition of FAK and Src, there is now significant apoptosis in the MYCN- as well as the MYCN+ isogenic cells lines. **D**, Apoptosis is detected biochemically by immunoblotting. After 24 hours of dual inhibition with AdFAK-CD and PP2, cell lysates are collected and Western blot performed (Materials and Methods) with antibodies that detect total PARP and total caspase 9. After 24 hours, treatment with AdFAK-CD leads to a loss of expression of total PARP and total caspase 9 in both cell lines, which becomes even more marked in the MYCN- cells with dual inhibition of Src and FAK after the addition of PP2 to the AdFAK-CD treatment (Fig. 5D, rows 1, 2, 3). Treatment with AdLacZ with or without PP2 did not affect total PARP or total caspase 9 in either cell line.