Focal adhesion kinase and paxillin : mediators of breast cancer cell migration

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CHAPTER 7

Discussion

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Tumor cell migration is an essential step in metastasis formation. Focal adhesions, the physical links between cells and ECM, are critically involved in the migratory process. Focal adhesions are composed of signaling (eg kinases), scaffolding and structural molecules. The molecular signaling pathways converging from focal adhesions are very complex, and an increased understanding of these pathways can contribute to the development of better options for treatment of metastatic disease. In the work described in this thesis, different experimental designs were used to study breast cancer cell migratory behavior.

Models to study tumor progression and metastasis formation

Numerous models have been generated to study primary tumor growth and secondary tumor formation *in vivo*. Many models make use of nude or severe combined immunodeficient (SCID) mice, to exclude the possibility that injected cells are rapidly cleared by the innate immune system. In humans, secondary tumors do not cause immunoreactivity, as the immune system does not consider them non-self. A model that mimics the human situation is the so-called syngeneic tumor model. In this model, cells isolated from animals are cultured and manipulated *ex vivo* to be injected back into the same species they were derived from. This model elegantly circumvents the immune system. The rat mammary adenocarcinoma cell line MTLn3 is an excellent model to mimic breast cancer and its treatment (1,2). In the early eighties, Neri and Welch isolated a founder cell line from a female Fischer 344 rat (3). Injection of these cells into the mammary fat pad of another Fischer 344 rat resulted in lung metastases. These metastases were isolated and cultured *ex vivo*, resulting in the MTLn3 cell line. MTLn3 cells turned out to be highly metastatic, burdening the lungs with metastases within 3 weeks after injection into the mammary fat pads once more. However, after prolonged culturing, the MTLn3 cells eventually lost most of their metastatic potential (4). A possible explanation for the lack of lung metastases could be that, despite the syngeneicity of the model, circulating MTLn3 cells are rapidly cleared by cells of the immune system shortly after injection. Using an *in vitro* cytotoxicity test, we found that natural killer (NK) cells, but not T cells, kill MTLn3 cells. To confirm the potency of NK cells to kill MTLn3 cells *in vivo*, we depleted NK cells from Fischer rats by injecting a NK depleting antibody on 3 consecutive days prior to injection of tumor cells (5). Using this method, the amount of lung tumors increased from 2 to 130, indicating that indeed NK cells efficiently eradicate

MTLn3 tumor cells from the circulation. Temporary depletion of NK cells from Fischer 344 rats allows MTLn3 cells to escape immunosurveillance and allows them to invade the lungs. With this improved model, biological mechanisms of metastasis formation can be studied in a syngeneic environment. In chapter 3 and 4, we made use of this model to investigate the involvement of FAK in primary tumor growth, secondary tumor formation and sensitivity to the anticancer drug doxorubicin.

One drawback of this model is the fact that injection of the antibody on three consecutive days prior to tumor cell injection, can potentially introduce more variation into the experiments. Some animals may have more NK cells than others, and this may lead to a variable response to the injected antibody. Furthermore, the need for depletion of NK cells makes the experiment less practical and more time-consuming. Recently, an orthotopic mammary gland tumor/metastasis model was described that makes use of $Rag2^{-/-}\gamma c^{-/-}$ mice which lack NK cells altogether (6). In chapter 6, we have employed this model to explore the role of JNK-paxillin signaling in metastasis formation.

Focal adhesion kinase signaling in breast cancer progression and treatment

In chapter 3 the syngeneic MTLn3-Fischer 344 model was employed to study the role of FAK in breast cancer cell growth and metastasis formation. FAK is a critical tyrosine kinase involved in cell motility and is frequently overexpressed in human tumors. First, an MTLn3 cell line was established that conditionally expresses FRNK, an inhibitor of FAK function, in a doxycyline-inducible manner (MTLn3-tetFRNK cells). When FRNK was expressed in these cells, it inhibited the *in vitro* localization of FAK at focal adhesions, and as a consequence FAK phosphorylation at focal adhesions was diminished. Although several studies show that inhibition of FAK negatively affects cell survival (2), expression of FRNK in MTLn3-tetFRNK cells did not result in increased apoptosis nor did it affect anoikis, most probably due to low expression levels. FRNK expression did attenuate attachment and spreading of MTLn3 cells, and it reduced their ability to migrate as studied in a wound healing assay. These findings are in agreement with several studies on the *in vitro* effects of FRNK (7-9) and indicate that FRNK expression could also affect the behavior of MTLn3 cells *in vivo*. When we used the syngeneic model described above to study the effects of FAK inhibition on primary tumor growth and experimental lung metastasis formation, we found that:

1) FAK is important in primary breast tumor growth; 2) FAK is essential in experimental lung metastasis formation; 3) FAK is required in the early phase of metastasis formation and 4) inhibition of FAK by the expression of FRNK does not cause dormancy in the tumor cells.

One of the major problems in the treatment of distant metastases is the fact that they often acquire resistance towards chemotherapy. This is due to increased proliferation and cell survival pathways and/or suppression of apoptotic signaling. As shown in chapter 3, and by others, FAK is implicated in cancer progression and survival signaling of (breast) tumor cells.

In chapter 4, we investigated the involvement of FAK in the sensitivity of primary tumors and experimental metastases towards doxorubicin in the syngeneic MTLn3-Fischer344 model. When FAK function was inhibited by the expression of FRNK *in vivo* (by doxycyline treatment of rats injected with MTLn3-tetFRNK cells), primary tumors were more susceptible to killing by doxorubicin, resulting in a significant decrease in primary tumor growth and size. Similarly, expression of FRNK also sensitized secondary tumors to doxorubicin treatment: less metastases were found in the lungs of animals treated with both doxycycline and doxorubicin, compared to animals treated with doxycycline only.

In an attempt to explain the FRNK-associated sensitization towards doxorubicin, we performed genome-wide expression profiling on MTLn3-tetFRNK cells. Gene ontology (GO) pathway analysis revealed that, among others, the pathway *regulation of transcription* was differentially expressed after FRNK expression. One of the genes found to be downregulated was Fra-1 (Fos-related antigen-1), a member of the activator protein-1 (AP-1) family of transcription factors. Because Fra-1 is activated in multiple types of cancer and Fra-1 gene ablation can suppress the invasive phenotypes of many tumor cell lines (10) (11), it is a likely candidate for involvement in the observed effects. Indeed, when Fra-1 was knocked down in MTLn3 cells by siRNA, focal adhesion dynamics, cell spreading and migration were negatively affected. The role of Fra-1 in susceptibility towards doxorubicin was examined next. Knockdown of Fra-1 facilitated doxorubicin-induced cell death and complimentarily, overexpression of Fra-1 rescued cells in which FAK was knocked down from doxorubicin-induced cell death, while it did not affect apoptosis in cells in which FAK was expressed normally.

Collectively, these data support a model in which increased FAK signaling in tumor cells is coupled to Fra-1 gene expression, providing cells with survival advantages and protection against doxorubicin. Fra-1 expression is correlated with the acquisition of a mesenchymal phenotype in epithelial cells and overexpression of Fra-1 in carcinoma cells of epithelioid origin greatly enhances motility and invasiveness (12). MTLn3 cells also have mesenchymal characteristics and we speculate that the metastatic potential and possibly even the drug-resistant features of these cells have an underlying FAK-Fra-1 signaling axis.

Focal adhesion kinase as an anticancer drug target

The findings obtained in chapter 2, 3 and 4 suggest that FAK is a potential target for the development of novel anti-metastasis drugs (13,14). Besides the expression of dominant-negative mutants such as FRNK, two other strategies to influence FAK (signaling) *in* vivo are conceivable: pharmacological intervention using small molecule FAK inhibitors and downregulation of FAK levels using short interfering RNA or antisense oligonuceotides.

Several FAK-selective or dual-specific small molecule inhibitors (including PF-562,271 and TAE226) have been developed and studied and some of them are currently being tested in clinical trials (15,16). For example, PF-562,271 is a potent ATP-competitive reversible inhibitor of FAK and the related kinase Pyk2 and is currently in phase I clinical trials (15). PF-562,271 shows antitumor efficacy *in* vivo in several human s.c. xenograft models including colon; breast; prostate; pancreatic; and hepatocellular carcinomas by decreasing the phosphorylation status of FAK (17). It is interesting to consider opportunities of combining novel small molecule FAK inhibitors with existing chemotherapeutics or molecular therapies. Combination of PF-562,271 with the antiangiogenic compound sunitinib, for example, shows synergistic effects on the inhibition of tumor growth (18).

FAK-specific antisense oligonucleotides and siRNAs can serve as useful tools to reduce the expression of endogenous FAK in cancer cells. FAK expression was greatly reduced in A549 adenocarcinoma cells by treatment with FAK antisense oligonucleotides and this resulted in inhibition of downstream signaling to JNK, as well as a decrease in cell migration and invasion through Matrigel (19). In murine 4T1 breast canrcinoma cells, FAK-specific short hairpin RNAs resulted in decreased FAK levels and inhibition of invasion. Importantly, this was reflected

by inhibition of lung metastasis formation in a syngeneic tumor model (20). The therapeutic potential of siRNAs is dependent on the stability *in vivo* and correct delivery at tumor sites. Delivery of FAK-specific siRNA in neutral liposomes was shown to be highly effective in reducing *in* vivo FAK expression as well as tumor weight in nude mice injected with human ovarian cancer cell lines (21). The studies in which siRNA or antisense oligonucleotides were used to abrogate FAK expression were all successful in doing so. However, the resulting phenotypes were not always similar. This could indicate that the type of cancer and the biological setting of the tumor (eg. microenvironment) may play important parts in determining the response to loss of FAK expression. This could complicate the applicability of siRNA or antisense oligonucleotides as therapeutic strategies.

Paxillin in microtubule interference-induced cytoskeletal changes

Loss of adherence and gain of migratory capacity are important hallmarks of cancer cell invasiveness. The dynamics of cell adhesion involve a constant remodeling of the cytoskeleton, and these dynamics are a requisite for cellular movement. Drugs that interfere with the microtubule cytoskeleton not only cause cell cycle arrest and apoptosis, but also alter the cytoskeletal network (22). In chapter 5, we have used the microtubule-interfering agent vincristine as a model compound to study the impact of cytoskeletal changes in breast cancer cells. Vincristine belongs to the family of the vinca-alkaloids, drugs used to treat several cancer types (23,24). Vincristine has been shown to cause cell cycle arrest and apoptosis in a variety of cell lines, and in recent years many efforts have been made to unravel the signaling pathways that mediate the biological activities of vincristine and other microtubule-interfering agents (25-28).

In MTLn3 cells, vincristine causes cell cycle arrest, cytoskeletal changes and apoptosis. We show that the induction of cell contractility, but not cell cycle arrest, is dependent on JNK activation. Furthermore, JNK is localized to focal adhesions and the focal adhesion adaptor protein paxillin is affected in a JNK-dependent way by phosphorylation and another yet unidentified modification upon treatment with vincristine. In our model system, paxillin functions as a downstream effector of JNK: paxillin is not required for JNK activation, though it is essential for the subsequent changes in contractility and focal adhesion dynamics. This JNK-paxillin activation occurs upstream of ROCK-dependent actin/myosin-mediated cell contractility: exposure to vincristine induces phosphorylation of MLC in

MTLn3 cells, and this phosphorylation can be prevented by either inhibiton of JNK or knockdown of paxillin. Our data indicate that focal adhesion signaling plays an important part in the effects of anticancer drugs *in vitro*; these findings could also have implications for the biological outcome of cancer treatment with microtubule-interfering agents.

JNK-paxillin signaling in the regulation of tumor cell migration

Activation of proteins by post-translational modifications represents an important cellular mechanism in regulating most aspects of biological organization and control. The complexity of protein modification includes phosphorylation and dephosphorylation of proteins in different pathways. Adaptor proteins play an important part in the convergence of signals, as they enable the approach of signaling partners into close proximity, thereby allowing post-translational modifications to occur. The focal adhesion-associated adaptor protein paxillin contains a great deal of phosphorylation sites including tyrosine, threonine and serine residues (29). Paxillin controls cell motility by regulating focal adhesion dynamics (30,31) and thus plays an important role in cell motility processes. One of the recently identified kinases that phosphorylate paxillin, in association with growth factor EGF-induced cell migration, is JNK (32,33). EGF signaling is of great importance in breast cancer biology as well as in the tumor metastasis process of MTLn3 cells (34). Furthermore, the levels of serine 178-phosphorylated paxillin are substantially increased in human hepatocellular carcinomas (HCCs), and phosphorylation of paxillin Ser178 by JNK is required for HCC cell migration (35).

In chapter 6 we have explored the role of paxillin serine 178 phosphorylation by JNK in EGF-induced signaling and breast tumor cell behavior. Expression of a paxillinS178A mutant significantly reduced EGF-induced cell migration and decreased activation of PI3K/AKT and ERK signaling. In an orthotopic mammary gland tumor/metastasis mouse model, phosphorylation of paxillin at serine 178 by JNK was essential for efficient metastasis of MTLn3 cells to the lungs. Tyrosine kinase receptor EGFR was found to be severely downregulated in S178A mutant-expressing cells, at the protein level as well as the mRNA level, and re-expression of EGFR restored tumor cell migration and lung burdening capacity. In summary, in MTLn3 cells, binding of EGF to its receptor

EGFR rapidly triggers activation of JNK, leading to the phosphorylation of paxillin at serine 178. This facilitates adhesion turnover and promotes cell migration.

Paxillin as an anticancer drug target

Paxillin's function as an adaptor protein makes it an interesting target to consider for anticancer drug design. Stopping paxillin from binding to certain partners could lead to inhibition of very specific processes or signaling routes. For example, small molecules could be designed that inhibit the phosphorylation of paxillin at serine 178 by JNK. As this phosphorylation event seems specifically involved in tumor cell migration, this may well be a feasible direction to look for anti-metastasis drugs. On the other hand, the complexity of paxillin signaling makes the specificity of inhibition very challenging, not in the least because of the abundancy of binding partners and their potentially redundant functions. Furthermore, the exact role of paxillin in tumor progression is still poorly understood, and contradictory results have been obtained in studies that investigated the expression of paxillin in human cancers (36-40). Considering these difficulties, it will be a challenge to target paxillin as an anticancer agent at this time. More research into the downstream signaling pathways may improve the potentiality of paxillin in anticancer drug development in the near future.

Future perspectives

Focal adhesions contain more than 150 components, enabling over 650 interactions (41). It will be of great importance to elucidate, even further, the relevance of each component or group of components in different model systems. In this way, more insight can be obtained into the complex signaling involved in different aspects of breast cancer progression. Because of their critical involvement in cell migration and thus in metastasis formation, focal adhesion-associated proteins remain important potential anticancer drug targets. It is very conceivable that for every different situation (including differences between patients, origins of the primary cancer, basal levels of involved proteins etc.) a different therapeutic approach will be necessary.

The work described in this thesis has employed various tools, both *in vitro* and *in vivo*, to study the role of focal adhesion-derived signaling in breast tumor cell behavior. We have shown that FAK and paxillin are key regulators in processes that define metastasis formation: adhesion, survival and migration.

Furthermore, we have discussed their potential as targets for anticancer drugs. Hopefully, the gained knowledge from this work and from future research will lead to an increased understanding of the complex signaling pathways that define cancer metastasis formation and possibly to the development of new, promising drugs to help combat the disease that affects women's lives all over the world.

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