Nucleophosmin-anaplastic lymphoma kinase (NPM–ALK) is a tyrosine kinase oncogene responsible for the pathogenesis of the majority of human ALK-positive lymphomas. We recently reported that the Rac1 GTPase in anaplastic large-cell lymphoma (ALCL), leading to Rac-dependent formation of active invadopodia required for invasiveness. Herein, we went further into the study of this pathway and used the inhibitor of Rac, NSC23766, to validate its potential as a molecular target in ALCL in vitro and in vivo in a xenograft model and in a conditional model of NPM–ALK transgenic mice. Our data demonstrate that Rac regulates important effectors of NPM–ALK-induced transformation such as Erk1/2, p38 and Akt. Moreover, inhibition of Rac signaling abrogates NPM–ALK elicited disease progression and metastasis in mice, highlighting the potential of small GTPases and their regulators as additional therapeutic targets in lymphomas.

Blood Cancer Journal (2011) 1, e21; doi:10.1038/bcj.2011.19; published online 3 June 2011

Keywords: anaplastic lymphomas; NPM–ALK; dissemination; Rac1 GTPase

Introduction

Translocation of the gene encoding the anaplastic lymphoma kinase (ALK) located on chromosome 2p23 results in the expression of chimera proteins (about 17 characterized so far) associated with various cases of non-Hodgkin’s lymphomas with a high grade of malignancy. The most frequent rearrangement, a t(2;5) (p23;q35) reciprocal translocation, fuses the N-terminal portion of nucleophosmin 1 (NPM) to the C-terminal portion of ALK that contains the catalytic domain to produce the NPM–ALK fusion protein, which constitutes tyrosine kinase activity accounts for transformation. \(^1,^2\) The majority of ALK translocations leads to hematological malignancies of the anaplastic large-cell lymphoma subtype (ALCL), but cases of diffuse large B-cell lymphomas and myeloproliferative leukemia have been described. \(^3,^4\) ALK is also expressed in a subset of primary solid cancers, either as the result of translocation or as a full-length intact or mutated protein. It has been found in inflammatory myofibroblastic tumors, non-small lung cancers, melanoma, breast carcinoma and neural tumors (neuroblastosoma, neuroectodermal tumors or glioblastomas). \(^1^,^2,^3,^4,^5\) Several groups have undertaken the study of molecular factors involved in ALK-dependent tumorigenesis. Many modulators, downstream effectors and associated oncogenes were identified and characterized. NPM–ALK functions as a scaffold that recruits a ‘signalosome’ of more than 40 proteins, involved in activation of proliferative and antiapoptotic responses, which have been extensively studied. \(^5^,^6\) The majority of ALK (+) ALCL are diagnosed at advanced III and IV stages, displaying systemic disease with a high propensity for extranodal dissemination, especially in the skin and soft tissues such as the liver, lung or spleen, clearly identifying ALK (+) malignancies as very invasive neoplasms. \(^2^,^7,^8\) Understanding the synergy between ALK and its signaling partners should facilitate the design of innovative therapeutic strategies.

Recently, regulators of cytoskeleton dynamics such as the Rho GTPases Rac1 and its activator the guanine-nucleotide exchange factor (GEF) Vav3, p130Cas and pp60 c–c expressed in anaplastic large-cell lymphomas are known to be implicated in NPM–ALK-dependent invasion. \(^8^,^9,^12\) Ambrogio et al. \(^1^3\) demonstrated that the Vav1/Cdc42 signaling cascade regulated NPM–ALK (+) cells survival and migration. Rho GTPases mediate many aspects of cell biology including proliferation, regulation of the apoptosis/survival balance, polarity, adhesion, membrane trafficking and motility. \(^1^4\) The high incidence of overexpression of some of them (RhoA, RhoC, Rac1, Rac3 and Cdc42) or their regulators in human tumors suggests that GTPases are important in the carcinogenesis process. \(^1^5\) Targeting Rho GTPases in mice has allowed to better understand their physiological role in vivo. \(^1^6\)

Our previous studies demonstrated the implication of Rac1 in mediating NPM–ALK-dependent invasion of ALCL cells. \(^9\) Importantly, we unraveled a central role for Rac in the structuration of functional invadopodia by allowing secretion of the metalloproteinase MMP-9 and the establishment of the CD44/MMP-9/Hsp90 complex that drives extracellular matrix degradation and 3D migration of NPM–ALK (+) cells in vitro. \(^1^7\) Herein, we sought to investigate whether inhibition of Rac by NSC23766, a small molecule inhibitor demonstrated to reproduce the effects of genetic invalidation of Rac1 and Rac2 in mice, could affect the tumorigenicity of NPM–ALK (+) cells and the development of the disease in a xenograft model of ALCL and in NPM–ALK-expressing transgenic mice.

Materials and methods

Reagents and antibodies

Cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). WHI-154 and NSC23766 were from Calbiochem (San Diego, CA, USA). Aprotinin and leupeptin were from Euromedex (Soufflwiesheim, France). All other chemicals were from Sigma-Aldrich (St Louis, MO, USA). The following antibodies were used in this study: ALK (Sp8; Abcam, Cambridge, UK), B220/CD45R (RA3-6B2; AbD Serotec, Colmar, France), Rac1 (23A8; Upstate Biotechnology, Lake Placid, NY, USA), phospho-ALK-Y1604 (recognizes Y664 on NPM–ALK),...
phospho-p38-T180/Y182, p38, phospho-Erk1/2- T202/Y204, Erk1/2, phospho-STAT3-Ty205, STAT3, phospho-Akt-S473, Akt were all from Cell Signaling Technologies (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Promega (Madison, WI, USA).

Mice
The generation of tTA/pBIL-NA transgenic mice expressing NPM–ALK under the tetracyclin regulatory system was previously described. Expression of the NPM–ALK transgene was obtained by removing doxycycline (100 μg/ml) from the drinking water. Animals used in the experiments were littermates. Female SCID (C.B-17-Prkdcscid/lecrCr) mice obtained from ‘Centre d’Elevage Janvier’ (Le Genest St Isle, France) were used at age 7–12 weeks. Mice were housed in the animal facility of INSERM U1043 (Purpan Hospital, Toulouse, France) under specific pathogen-free conditions. Experiments were conducted according to animal protocols approved by the INSERM.

Cell culture
Human ALCLs cell lines Karpas 299 and Cost were cultured in Iscove-modified Dulbecco medium (IMDM) supplemented with 15% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% carbon dioxide. Cost ALCL cell line19 was obtained from G Delsol (CRCT, Toulouse, France). Karpas 299 ALCL cells were obtained from DSMZ (German Collection of Microorganisms and Cell Culture, Braunschweig, Germany). Cell lines were checked twice a month for Mycoplasma (MycoAlert Mycoplasma detection assay, Lonza, Basel, Switzerland).

Immunoblotting
Total cellular proteins were extracted with 50 mM Tris-base pH 8, 150 mM NaCl, 5 mM EGTA, 1% Nonidet P-40, 1 mM PMSF, 50 mM NaF, 1 mM Na3VO4, 10 μg/ml leupeptin and 2 μg/ml aprotinin. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by western blotting on Immobilon-P membranes (Millipore, Billerica, MA, USA) with appropriate antibodies. Immunoreactive bands were detected by chemiluminescence with the SuperSignal detection system (Pierce Chemical Co, Rockford, IL, USA).

Matrigel invasion assays
The Matrigel invasion assay measures the ability of ALCLs to invade a dense Matrigel matrix (BD Biosciences, Rockville, MD, USA) that mimics the basement membranes. A total of 2 × 105 overnight serum-starved ALCLs cells were labeled for 90 min with 0.5 μM CellTracker Orange CMTMR (Invitrogen). After washes, cells were seeded in labtek chambers and overlaid with Matrigel at 5 mg/ml, +/− 40 μM NSC23766. After 90 min at 37 °C, medium containing 15% fetal calf serum and 1 μg/ml SDF1α was added. After 24 h, cells were analyzed by confocal microscopy (LSM510. Carl Zeiss, Oberkochen, Germany) using a 20 × objective. Optical z sections were taken every 2.5 μm starting from the base and extending 100 μm into the Matrigel. To quantify invasion, CMTMR fluorescent cells in the sections above 20 μm were counted and then divided by the sum of cells in all the z sections. Data represent the analysis of ≥ 200 cells per condition. Three-dimensional imaging and cell quantification were realized using the Imaris 6.4.2 software (Bitplane AG, Zurich, Switzerland).

In vivo tumorigenicity assays
Karpas 299 cells were centrifuged and resuspended in 150 μl of phosphate-buffered saline (PBS) before being injected subcutaneously into SCID mice (6 × 105 cells per animal). Tumors were let to develop for 7 days, then mice were treated twice a day by intraperitoneal administration of PBS or 2.5 mg/kg of NSC23766 for 9 additional days. Tumor development was assessed by calliper measurements. Volume was calculated according to the formula: length (mm) × thickness (mm) × width (mm) × 0.52. Results are expressed as ratio of the tumor volume at each time point over the volume before treatment. On day 10 post-treatment, mice were killed and dissected. Tumors and organs were fixed in 4% formalin and embedded in paraffin. Transgenic tTA-pBIL-NA mice (3-weeks old) were treated with NSC23766 for 14 days as described above.

Immunohistochemistry
Sections of tissues were incubated with the following antibodies: ALK (Sp8), or B220/CD45R. Development was described previously. Antibody binding was detected using the Impress Rabbit Kit (Vector laboratories, Burlingame, CA, USA). Sections were observed with a Leica DMR microscope (Leica, Solms, Germany) at the indicated magnification.

Statistical analysis
The mean ± standard error was calculated for each data point. Data are representative of three to five independent experiments. Differences between groups were analyzed by the Student’s t-test. In all cases, P<0.001.

Results
Rac controls transforming properties of NPM–ALK (+) cells in vitro
We had previously shown that expression of the dominant negative Rac1-T17N mutant abrogated invasion of NPM–ALK (+) fibroblasts. We used the Rac small-molecule inhibitor NSC23766 to analyze its effects on NPM–ALK-induced invasiveness in vitro in NPM–ALK (+) ALCL cell lines Cost (small-cell variant) and Karpas 299 (common type)20. Overnight treatment with 40 μM NSC23766 resulted in the blockade of Rac1-GTP loading, while RhoA and Cdc42 were unaffected (Supplementary Figure 1).

NPM–ALK has been reported to affect cell morphology, adhesion and migration in various cell types. In ALCL cells invading 3D-Matrigel, NPM–ALK transformation led to the formation of dynamic membrane protrusions around the cellular body, which display polarized motile actin-rich membrane protrusions towards a SDF1α gradient. We then proceeded to evaluating whether NPM–ALK-dependent invasion was affected by NSC23766 using the same experimental set-up. Figure 1 showed that treatment of Cost and Karpas 299 NPM–ALK (+) ALCL cells with 40 μM NSC23766 abolished invasion of 3D-Matrigel, demonstrating that pharmacological inhibition of Rac was similar to the classical dominant negative approach to block Rac-dependent cellular responses in vitro.

The oncogenic potential of ALK fusions relies on their hyperactive tyrosine kinase activity that activates interconnected signaling pathways common to many oncogenic kinases. For instance, resistance of ALK (+) cells to apoptosis relates to JAK3/STAT3 and PI3 kinase/Akt pathways, while increased proliferation is accounted for by the activation of Src family
kinases, Grb2/Sos/Ras, MAPK (Erk1/2, JNK and p38 (ref. 5). As expected, Figure 2 demonstrated that those signaling modules were strictly dependent on ALK kinase activity as treatment of Cost and Karpas 299 with the WHI-154 inhibitor led to their inactivation. Because some of those NPM–ALK targets were described as Rac downstream effectors, we evaluated their status of phosphorylation/activation upon NSC23766 treatment. We observed a drop in p38, Erk1/2 and Akt phosphorylations that was similar to that obtained with ALK inhibition (Figure 2). Importantly, NPM–ALK and STAT3 activation were spared, demonstrating that only some of the pathways known to trigger ALK-dependent transformation were under the regulation of Rac.

Rac drives invasion of NPM–ALK (+) ALCL in vivo

The main advantage of using a selective pharmacological inhibitor is its capacity to be used in animal models. To evaluate the impact of Rac1 signaling on in vivo transforming properties of NPM–ALK, Karpas 299 cells were subcutaneously engrafted in immunocompromised SCID mice. NPM–ALK (+) cells gave rise to rapidly growing tumors within 7 days. Thereafter, animals were injected twice a day with intraperitoneal doses of 2.5 mg/kg NSC23766 (NSC23766 mice) or PBS (PBS mice).

We observed that NSC23766 treatment blocked tumor development (volume = 34 ± 12 mm³, 9 days post-treatment) in contrast to PBS-injected mice in that tumor growth still progressed (volume = 436 ± 14 mm³, Figures 3a and b). The tumors from the PBS mice had indistinct margins and several foci of necrosis, typical of fast developing neoplasia, whereas tumors from SCID mice were small with distinct margins. Immunohistochemistry demonstrated that both types of subcutaneous tumors were formed by Karpas 299 displaying the characteristic phenotype of large NPM–ALK (+) cells (Figure 4).

Macroscopic observations of the organs after autopsy revealed marked splenomegaly and lymphadenopathy suggesting a dissemination of Karpas 299 cells from the original subcutaneous tumor in PBS mice. Spleens of PBS mice reached 2.3 ± 0.3 mm, while it was reverted to normal size of 1.4 ± 0.1 mm in NSC23766-treated mice (Figures 3c and d). To better assess the extent of dissemination, immunohistochemistry with anti-ALK antibody was performed on tissue sections from subcutaneous tumor, lymph node (LN), spleen, lung and liver, which correspond to sites of metastasis usually found in human disease.11 Figure 4 (upper panel) showed that Karpas 299 cells populate the lung, liver, lung node and spleen of PBS mice. However, NSC23766 treatment (tumor size ± s.e.m.) effectively prevented the dissemination of Karpas 299 cells to the organs as no positive immunostainings were observed in spleen, lung and liver sections of Karpas 299 tumors treated with NSC23766 (Figure 4, lower panel). Inhibition of Rac downregulates growth and spreading of xenografted NPM–ALK (+) Karpas 299 tumors. Karpas 299 cells were subcutaneously injected into SCID mice. Tumor development was allowed for 7 days, before 9 days of treatment with NSC23766 (two intraperitoneal injections per day, 2.5 mg/kg). (a) Black and white pictures of subcutaneous tumor masses after 9 days of treatment, dotted circle delineate the tumors. (b) Tumor development was assessed by calliper measurements, data are presented as the percentage of the tumor volume at the indicated time over the size at D0 (when treatment was started). Data are from groups of five (PBS) and seven (NSC23766) mice. (c) Spleen enlargements of PBS- or NSC23766-treated littermates are shown. (d) The histogram represents the mean ± s.e.m. of spleen size.
were found in all tested organs in control PBS mice, demonstrating that subcutaneously xenografted ALCL cells were highly invasive. Conversely, none of the NSC23766-injected mice showed signs of ALK-staining in distant organs, while about a fourth of them (3 out of 11 mice) still showed a slight LN infiltration on the side of tumoral engraftment (Figure 4, lower panel). These data indicate that the Rac pathway has a central role in regulating tumoral growth, as well as invasiveness of NPM–ALK (+) ALCL cells in xenografted mice models.

Pharmacological inhibition of Rac impairs lymphoma development in transgenic mice
To better assess the impact of inhibiting Rac-dependent signaling on ALK (+) pathogenesis, we took advantage of a recently developed conditional transgenic mouse model of ALK-induced lymphomagenesis that uses the tetracycline regulatory system under the control of the EpSra enhancer/promoter. In this model, the expression of NPM–ALK induces B-cell lymphoma/leukemia and the development of skin keratoacanthoma-like lesions resulting from aberrant expression of the transgene in keratinocytes. Even though it does not reproduce faithfully the human disease, this model represents a major tool to investigate in vivo molecular mechanisms involved in ALK-associated disorders, and to test putative therapeutic targets. On expression of NPM–ALK by retrieving doxycycline from the drinking water at birth, mice develop the disease around 3 weeks of age. It is characterized by a typical skin disorder that affects snout and paws (Figure 5a), growth defects (Figure 5b) and general lymphoid malignancy with marked splenomegaly and lymphadenopathy (Figure 5c), with death happening between 4 and 5 weeks. Mice (3-weeks old) that just showed the first signs of the disease were treated either by injections of NSC23766 or PBS as a control. At 5 weeks of age, although control littermates were dying, NSC mice were physically active, gained weight and exhibited a regression of the skin lesions within a few days of treatment (Figures 5a and b). Accordingly, LNs and spleens regained their normal size (Figure 5c). Regression of the lymphoid disease was confirmed by immunohistochemistry. Figure 6 showed that the architecture of the LNs of iTA/pBIL-NA mice was obliterated by infiltration of tumoral cells of B origin in this model as shown by the positive staining of the CD45R/B220 antibody. These cells were also expressing NPM–ALK. Inhibition of Rac by NSC23766 restored the normal architecture of the LNs and markedly reduced the infiltration by NPM–ALK/B220-positive lymphoma cells, which became similar to what was observed when the oncogene was repressed by doxycycline administration (Figure 6). Analysis of the peripheral organs (spleen, lung and liver) demonstrated a marked regression or disappearance of infiltrating lymphoma cells (Figure 7). Altogether, these data demonstrate the major role of Rac in lymphomagenesis and dissemination of NPM–ALK malignancies. The effects of NSC23766 provide a rationale for targeting small GTPases in ALK-dependent cancers.

Discussion
If the roles of Rho GTPases have been well described in the physiology of adherent cells and their involvement in solid tumors is documented, a lot remains to be study regarding their part in hematopoiesis and hemopathies. Specific pathways regulated by GTPases were shown to be crucial for hematopoietic stem cells, T- and B-cells maturation and functions. Small GTPases are known effectors of T- and B-lymphocytes where they partake in activation through cytoskeleton rearrangements, polarization and formation of the immunological synapse required for efficient lymphocyte response. These observations account for the deregulations of Rho GTPases associated with hematological malignancies such as leukemia, non-Hodgkin’s lymphomas or ALCL as well as immune pathologies. Even though they lack the TCR/BCR and most of the signaling relays of stimulated lymphocytes, ALCL display the phenotype of activated T- or B-cell, and NPM–ALK oncogenesis involves pathways shared with lymphocytes activation. In this context, we and others produced a series of papers demonstrating the involvement of Rac and Cdc42 in cytoskeleton rearrangements and proliferation that drive invasiveness of ALK (+) ALCL, thereby opening new therapeutic perspectives. In this study, we demonstrated that NSC23766 treatment resulted in a blockade of ALCL-derived cell lines invasion of 3D-Matrigel, identifying Rac as a major relay of NPM–ALK-dependent invasive properties.

NSC23766 emerged as a powerful tool as it displays no significant toxicity in mice even after weeks of daily administration. Therefore, we treated mice that had developed subcutaneous tumors formed by the human NPM–ALK (+) ALCL cell line Karpas 299 by daily doses of NSC23766. Even though we did not observe any regression, the growth and spreading of the tumors harbored by NSC23766-treated mice...
were totally blocked compared with control animals. A similar experiment was conducted by the group of Chiarle who investigated the role of Cdc42 in the growth and maintenance of subcutaneously engrafted ALK (+) tumors. In this study, they identified Cdc42 as an important regulator of tumor development. Inhibition of Cdc42, either by small hairpin RNA or by the Secramine A inhibitor lead to cell death over time. We did not detect any apoptosis in our models (not shown). This could be explained by the persistent STAT3 activation under NSC23766, even though MAPK family members (p38 and Erk1/2) and Akt NPM–ALK-dependent activation was abolished. As a target of NPM–ALK, STAT3 has a major role in ALCL tumorigenesis by increasing the transcription of antiapoptotic factors and cell-cycle regulators such as BCL-XL, cyclin D3 or C/EBPβ, to increase survival and proliferation. 24 Therefore, its inactivation might be required to induce apoptosis of NPM–ALK (+) cells.

We then took a step forward by using newly developed conditional transgenic mice for ALK-induced lymphomagenesis under the tetracycline regulatory system. In this model as in many

Figure 5 Macroscopic observation of the regression of the disease of NSC23766-treated NPM–ALK-expressing tTA/pBIL-NA transgenic mice. Expression of the NPM–ALK transgene was induced by removing dox from the drinking water of newborn transgenic mice. After 3 weeks, mice were treated twice a day by intraperitoneal injection of PBS or 2.5 mg/kg of NSC23766. (a) Black and white pictures of littermate mice showing the decrease in size and the development of keratoacanthoma-like lesions on the snout and paws of PBS control mice. NSC23766 treatment reverses the phenotypes. (b) Measurements of the size increase generated by NSC23766 administration. (c) Rac inhibition reverted the typical spleen and LNs enlargements observed in transgenic mice.

Figure 6 Rac inhibition induces histophatological changes of lymph nodes of NPM–ALK transgenic mice comparable to oncogene silencing by re-administration of doxycycline. A 3-weeks-old tTA/pBIL-NA mice expressing NPM–ALK were treated for 14 days with NSC23766 as previously described. Mice were killed and LNs harvested, fixed and paraffin embedded for immunohistochemical analysis with the anti-ALK (Sp8) and the anti-B220 (B-lymphocytes) antibodies. The architecture of LN from PBS mice with a fully developed disease was obliterated by lymphoma cells positive for both B220 and ALK. Upon NSC23766 administration (NSC23766 mice), the LN progressively recovers a normal architecture, with a decrease of ALK (+) cells and an increase in B- (B220 positive) and T-cells (blue areas) similar to dox treatment (dox).
others that were developed with NPM–ALK, the expression of the oncogene causes B-cells lymphomas. It offers a powerful tool to investigate in vivo the mechanisms associated with ALK-induced transformation and therapeutic options, which was illustrated as a proof of principle by the use of the ALK inhibitor PF-02341066.18 Administration of NSC23766 to transgenic mice that were starting to develop the disease blocked the development of the lymphoid malignancy and significantly reduced the keratoacanthoma-like lesions (Supplementary Figure 2) observed in this model. Metastasis of target organs was totally abrogated, demonstrating that treatment with this first-generation Rac inhibitor was of major interest in ALK-driven malignancies. We tested several modes of administration of the drug. Intraperitoneal injections were conducted in parallel to delivery with Azlet osmotic pumps (one pump, 50 μM NSC23766). Both treatments gave the same results, the intraperitoneal administration being slightly more efficient, probably due to the short life-time of NSC23766 that is likely inactivated by remaining at the mouse body temperature over 2 weeks when delivered through the pumps. It has to be noted that NSC23766 was already shown to limit the development of BCR-ABL (+) chronic myeloid leukemia, even in the case of cells that were resistant to the Abl inhibitor classically used in therapy, imatinib. Thomas et al.23 demonstrated that co-administration of low doses of NSC23766 and imatinib significantly affected the development of the disease. These data clearly suggest that targeting Rac is a valid option for the treatment of various hematological malignancies. Indeed, the ALK inhibitor PF-02341066 (crizotinib) has just started to enter clinical trial to target the EML4-ALK kinase in non-small-cell lung cancer, and PF-02341066 (crizotinib) has just started to enter clinical trial to target the EML4-ALK kinase in non-small-cell lung cancer, and targeting Rac is a valid option for the treatment of various hematological malignancies.

To conclude, this work identified the Rac1 pathway as being important for the dissemination of the disease and establishment of metastasis in vivo. Our data together with the literature place Rho GTPases at the center of blood malignancies. It highlights the fact that the development of inhibitors of this family of proteins is a promising and valid option for therapeutic intervention in both lymphoma and leukemia.

Acknowledgements

We thank Dr T Al Saati and F Capilla (Experimental Histopathology Platform, IFR150-IFR BMT), Dr S Allart and D Sapédé (Imaging Facility, IFR150-IFR BMT) and the animal facility employees for their help. We are grateful to the members of Pr Payrastre’s group for helpful discussions. This work was supported by grants from the INSERM, ‘Ministère de la Recherche et de la Technologie’, ARC, INCa, ‘La Ligue contre le Cancer’, ‘Cancerpôle GSO’ and the ‘Pôle de compétitivité Cancer-Biosanté’ Fonds uniques interministériels des pôles de compétitivité’.

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