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4-Phenylbutyric acid: An AMPK agonist and mTORC1 inhibitor

4-phenylbutyric acid (4-PBA) is classified as an orphan compound by the National Cancer Institute and has been shown to inhibit the growth of certain types of cancer [1, 2]. This compound has been reported to function as a nitrogen scavenger used in the treatment of urea cycle disorders, a chemical chaperone, and a histone deacetylase (HDAC) inhibitor [3-6]. 4-PBA is relatively non-toxic at doses up to 550 mg/kg/day [7]. Additionally, whole body protein synthesis has been shown to be reduced in urea cycle disorder patients at 4-PBA concentrations within the safe non-toxic spectrum of the drug [6]. 4-PBA has also been shown to reduce endoplasmic reticulum stress which functions as an adaptive mechanism in certain cancers [8, 9]. For these reasons, we chose to investigate 4-PBA activities against mTORC1 signaling as an alternative to rapamycin therapy. Our results show that 4-PBA potently inhibits mTORC1 in glioblastoma, non-small cell lung carcinoma and colon carcinoma cell lines. Additionally, we found that treatment of these cells with 4-PBA results in the activation of AMP-activated kinase (AMPK) in an LKB1-dependent manner. However, because mTORC1 is still potently inhibited by 4-PBA in cell lines that lack LKB1, inhibition of mTORC1 by 4-PBA appears not to be mediated exclusively via AMPK. Our findings suggest that 4-PBA may be a pharmacologically useful alternative to rapamycin for inhibiting mTORC1 signaling in certain cancers. Nevertheless, the mechanisms by which 4-PBA inhibits mTORC1 require further investigation. Ongoing studies are addressing whether TSC2 and Rheb signaling to mTORC1 are affected by 4-PBA treatment and how the drug might regulate mTORC1 independently of AMPK.

1. Carducci MA, Nelson JB, Chan-Tack KM, Ayyagari SR, Sweatt WH, Campbell PA, Nelson WG, Simons JW. (1996) Phenylbutyrate induces apoptosis in human prostate cancer and is more potent than phenylacetate. *Clin Cancer Res*, 2, 379-87.
2. Svechnikova I, Almqvist PM, Ekstrom TJ. (2008) HDAC inhibitors effectively induce cell type-specific differentiation in human glioblastoma cell lines of different origin. *Int J Oncol*, 32, 821-7.
3. Brusilow SW, Finkelstien J. (1993) Restoration of nitrogen homeostasis in a man with ornithine transcarbamylase deficiency. *Metabolism*, 42, 1336-9.
4. Perlmutter DH. (2002) Chemical chaperones: a pharmacological strategy for disorders of protein folding and trafficking. *Pediatr Res*, 52, 832-6.
5. Jung M. (2001) Inhibitors of histone deacetylase as new anticancer agents. *Curr Med Chem*, 8, 1505-11.
6. Darmaun D, Welch S, Rini A, Sager BK, Altomare A, Haymond MW. (1998) Phenylbutyrate-induced glutamine depletion in humans: effect on leucine metabolism. *Am J Physiol*, 274, E801-7.
7. Samid D, Shack S, Sherman LT. (1992) Phenylacetate: a novel nontoxic inducer of tumor cell differentiation. *Cancer Res*, 52, 1988-92.
8. Hersey P, Zhang XD. (2008) Adaptation to ER stress as a driver of malignancy and resistance to therapy in human melanoma. *Pigment Cell Melanoma Res*, 21, 358-67.
9. Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, Gorgun CZ, Hotamisligil GS. (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science*, 313, 1137-40.

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Effect of doxycycline in ELT3 cells and angiomyolipoma xenografts

Background

Matrix metalloproteinases (MMPs) have been implicated in the pathogenesis of the cystic destruction in pulmonary lymphangiomyomatosis (LAM) in both *in vitro* and *in vivo* studies. Doxycycline inhibits the production of MMPs and some women with LAM use doxycycline as one case report suggested a possible benefit in the disease. However there have been no randomised controlled clinical trials of doxycycline for LAM and any mechanism of action is unclear.

Methods

We examined MMP-2 and -9 expression by quantitative real time PCR and zymography in doxycycline treated Eker rat derived ELT3 cells and angiomyolipoma derived tumour xenografts in nude mice. Proliferation and adhesion were examined by MTT reduction and a commercially available cytomatrix cell adhesion kit respectively. Xenograft tumour growth was measured over 60 days in doxycycline treated and control animals.

Results

ELT3 cells express MMP-2 mRNA and protein, MMP-9 mRNA was present but the protein was undetectable by zymography. Doxycycline treatment caused a dose dependent decrease in MMP-2 protein in ELT3 cells, with a 15% reduction in pro-MMP-2 at 10microg/ml. Paradoxically MMP-2 and -9 mRNA increased after doxycycline treatment. Doxycycline 50µg/ml decreased proliferation in ELT3 cells by 50% (n= 7, p= 0.0043) at 48 hours and was associated with a change in cell morphology. The effects on morphology and growth were reversible on doxycycline withdrawal. Doxycycline <25microg/ml did not have these effects. Both cell adhesion and detachment were decreased by doxycycline, again by doses ≥25µg/ml. In the xenograft model, doxycycline 30mg/kg/day (equating to a serum concentration of 2-3 µg/ml) had no effect on tumour growth or final tumour weight.

Conclusions

Doxycycline decreases MMP-2 expression at pharmacologic doses. Effects on cell proliferation, adhesion and detachment only occur at doses ≥25 microg/ml. These doses are likely to be supra-pharmacological as in humans doxycycline 200mg/day equates to a mean serum concentration of 4 µg/ml.

Debbie Clements, PhD
University of Nottingham

Chemokine receptor expression in lymphangioliomyomatosis and angiomyolipoma: role of the CXCR4 / SDF axis

Introduction

LAM has similarities with cancer including uncontrolled growth and metastatic behaviour. Recently chemokine receptors, long recognised for their role in recruitment of inflammatory cells, have been demonstrated to mediate cell homing, proliferation and survival in cancer cells. We examined the expression of chemokine receptors in LAM and angiomyolipoma tissue to determine whether they might also play a role in LAM pathology.

Results

We have detected expression of the chemokine receptor CXCR4 in LAM lung nodules and TSC-related AMLs by immunohistochemistry and RT-PCR. Treatment of low passage primary AML cells with CXCL12, the only known ligand for CXCR4, results in activation of AKT and p42/44 MAPK, but did not promote migration of the cells. There was, however, a small stimulatory effect on proliferation and resistance to staurosporine-induced apoptosis.

In a mouse xenograft model of angiomyolipoma, generated by subcutaneous engraftment of SV7 Tert AML cells, expression of CXCR4 was detected in the tumours by immunohistochemistry. In this model, tumour growth is inhibited by rapamycin, but treatment of the hosts with the CXCR4 antagonist AMD3100 alone did not significantly reduce tumour growth, nor was rapamycin plus AMD3100 more inhibitory than rapamycin alone.

Conclusion

Cells relevant to LAM pathology express the chemokine receptor CXCR4 and respond to CXCL12 by activation of AKT and p42/44 MAPK signalling pathways. The role of this signalling is unclear as downstream effects are modest. However small effects on proliferation and cell survival may prove significant over prolonged periods in vivo.

This study was funded by LAM Action and The LAM Foundation

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Matrilysin/MMP-7 expression in lymphangioleiomyomatosis (LAM)

Introduction

The mechanism of destruction of the lung parenchyma in LAM is unknown, although the activity of matrix metalloproteinases (MMPs) may be a contributing factor. Since pulmonary LAM cells and cells from renal angiomyolipomata (AML) of LAM patients appear to share a common origin, we sought to characterise MMP expression in AML tissue with the aim of extrapolating these data to the less readily available pulmonary LAM cells.

Results

We have isolated cells from renal angiomyolipomata of patients with tuberous sclerosis, and found relatively very high levels of expression of MMP-7 mRNA by quantitative RT-PCR. We also detect MMP-7 protein by immunohistochemistry in AML tissue (5/6 cases) and LAM lung nodules (6/7 cases), and by immunosorbent assay (ELISA) in primary AML cell culture supernatants. MMP-7 expression in primary AML cells is not sensitive to inhibition by rapamycin, nor is MMP-7 expression elevated in TSC2^{-/-} mouse embryonic fibroblasts or Eker rat derived ELT-3 cells.

Serum and BALF levels of MMP-7 were measured by ELISA in patients with LAM (serum samples: n=82, BAL samples n=15) and compared to levels in healthy volunteers (serum samples; n=36, BALF samples n=15). No significant differences were observed in BALF or serum levels of MMP-7 between patients with LAM and healthy volunteers (P>0.05). However, the serum MMP-7 levels of patients with LAM with lymphatic involvement (presence of adenopathy and/or lymphangioleiomyomas) were significantly higher than levels in patients without lymphatic involvement (P=0.037).

Conclusions

MMP7 is expressed in pulmonary LAM nodules and TSC-AML cells. Expression in primary AML cells is not sensitive to inhibition by rapamycin so does not appear to be a consequence of mTOR dysregulation. We hypothesize that MMP-7 activity in pulmonary LAM cells might contribute to cyst formation, either directly, or indirectly via activation of other MMPs.

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Renal and liver tumors in *Tsc2*^{+/-} mice, a model of tuberous sclerosis complex tumors, do not respond to atorvastatin, a HMG CoEnzyme A reductase inhibitor

Introduction

Mutations of the tumor suppressor gene, tuberous sclerosis complex 2 (*Tsc2*) results in activation of the G proteins, Rheb and RhoA, leading to activation of mTORC1. Statins, as inhibitors of prenylation have inhibitory effects on Rheb and RhoA that could potentially be therapeutic in disease states characterized by loss of *Tsc2* function, such as lymphangioliomyomatosis (LAM) and tuberous sclerosis complex (Can Res 2007;67:9878).

Methods

We tested atorvastatin as therapy for ENU-enhanced renal cystadenomas and spontaneous liver hemangioma in *Tsc2*^{+/-} mice. ENU-treated *Tsc2*^{+/-} mice were given atorvastatin (wt/wt 0.1%) for 1 month or 3 months (n = 8 each), prior to sacrifice at 6 mo; non-ENU treated *Tsc2*^{+/-} mice were given atorvastatin for 6 months (n = 10) prior to sacrifice at 12 mo. All treatment groups were compared to vehicle controls of identical genotype and a validated scoring system was used to assess tumors.

Results

The administration of ENU resulted in early tumor induction (cystadenoma) in the kidneys of *Tsc2*^{+/-} mice. Spontaneous renal cystadenoma and liver hemangioma were observed at the usual frequency in *Tsc2*^{+/-} mice. Immunohistochemistry revealed elevated levels of pS6 in renal tumors, consistent with loss of *Tsc2* function and activation of mTORC1. In both treatment groups, serum cholesterol, (ELISA) and levels of phosphorylated S6 (pS6; Western immunoblot) in healthy lung and liver extracts were significantly reduced in atorvastatin-treated mice compared to controls (> 50% each; p < 0.01). However, following atorvastatin treatment, no significant reduction in tumor size or tumor morphology (cystic:solid ratio) was observed for ENU-associated renal cystadenoma or spontaneous liver hemangioma compared to vehicle-treated controls. Additionally, in contrast to that observed in healthy tissue, no significant reduction in pS6 levels was observed in atorvastatin-treated tumors.

Conclusion

Our results indicate that although atorvastatin was effective as a HMGCR inhibitor, it not did inhibit the growth of ENU-enhanced renal cystadenoma or spontaneous liver hemangioma in *Tsc2*^{+/-} mice. These results suggest caution in considering statin use for treatment of TSC-associated lesions.

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Small GTPases Rac1 and RhoA modulate proliferation of cells with TSC2 dysfunction

Lymphangiomyomatosis (LAM) manifests by the abnormal growth of smooth muscle-like cells within the lung, which promotes the cystic destruction of the lung and loss of pulmonary function. LAM progression is associated with loss of function mutations of tumor suppressor genes *tuberous sclerosis complex 1 (TSC1)* and *TSC2*, which leads to constitutive activation of the mTOR/S6K1 signaling and abnormal cell proliferation. Previously, we reported that TSC1-dependent activation of RhoA GTPase is involved in aberrant proliferation of cells with TSC2 dysfunction. Because we also demonstrated that small GTPase Rac1 acts upstream of RhoA and inhibits RhoA activity in TSC2-null cells, we hypothesized that Rac1 play a role in proliferation of cells with TSC2 dysfunction. We found that PDGF, well known Rac1 activator, has failed to activate Rac1 in TSC2-null ELT3 cells suggesting that TSC2 loss modulates Rac1 activity. Interestingly, the overexpression of TSC1 in control NIH 3T3 cells also abolished PDGF-induced Rac1 activation suggesting that TSC1 also modulates Rac1 activity. To examine the relationship among TSC1, TSC2, and Rac1, we transfected TSC2-null ELT3 cells with TSC1 siRNA and found that TSC1 knock-down in the absence of TSC2 results in Rac1 activation, suggesting that TSC1/TSC2 complex modulates Rac1 activity. To directly examine the role of Rac1 in TSC2-related cell proliferation, we transfected TSC2-null ELT3 cells with GST-tagged constitutively active Rac1 mutant. We found that Rac1 activation significantly attenuated ELT3 cell proliferation by 22±1.58% compared to GST-transfected cells. Collectively, our data demonstrate that TSC1/TSC2 complex reciprocally modulate Rac1 and RhoA GTPase activities, and suggest that TSC1/TSC2-dependent modulation of Rac1 and RhoA is involved in abnormal proliferation of cells with TSC2 dysregulation.

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The cost-effectiveness of screening for lymphangioleiomyomatosis with high-resolution CT in women presenting with spontaneous pneumothorax

Introduction/Rationale

The average number of lifetime pneumothoraces in women with pulmonary lymphangioleiomyomatosis (LAM) who present with a sentinel spontaneous pneumothorax (SPTX) is 3.5. The diagnosis of LAM is typically delayed until after the second pneumothorax. Earlier diagnosis through high resolution CT (HRCT) scanning and treatment with pleurodesis could reduce the rate of recurrent SPTX, a major cause of morbidity for women with LAM. Although primary spontaneous pneumothorax (PSP) is the most common overall cause for apparent SPTX, PSP is considerably less prevalent in non-smoking women; the population subset that is enriched for patients with LAM. For non-smoking women, ages 25-54 years with SPTX, the predicted prevalence of LAM is 5%. We sought to determine if a strategy of HRCT screening for LAM followed by pleurodesis to prevent future SPTX is cost-effective in this population.

Methods

We constructed a Markov state-transition model to assess the cost-effectiveness of screening patients presenting with SPTX for LAM. Rates of SPTX and prevalence of LAM in given populations were derived from the literature. Costs of testing and treatment were extracted from 2007 Medicare data. We compared a strategy utilizing HRCT screening followed by pleurodesis for patients with LAM, versus no HRCT screening.

Results

In our base case analysis screening for LAM with HRCT is the most costly and most effective strategy with a marginal cost-effectiveness ratio of \$41,099 per quality adjusted life year gained. Sensitivity analysis showed that HRCT screening remains cost-effective in populations in which the prevalence of LAM is greater than 3.5%. The diagnostic utility of HRCT in LAM also significantly affected the results.

Conclusion

Screening for LAM with HRCT in non-smoking women age 25-54 that present with SPTX is cost-effective. Primary care and emergency medicine physicians are advised to screen for LAM with HRCT in this population.

Adem Kalender

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Metformin inhibits mTORC1 through an autonomous energy-dependent pathway, independent of TSC1/2 and AMPK

Dysfunctional-mTORC1 signaling is associated with a number of human pathologies due to its central role in controlling cell growth and proliferation. Regulation of mTORC1 is achieved by the integration of a number of multiple inputs, including those of mitogens, nutrients and energy. As a function of energy stress, or a decrease in the ATP:AMP ratio, acute control of mTORC1 is thought to be largely mediated by AMPK activation of the TSC1/2 tumor suppressor complex. Unexpectedly, we find that a number of agents that inhibit mTORC1 signaling by acute energy depletion, particularly the anti-diabetic drug metformin, act independently of TSC1/2 and, even more surprisingly independently of AMPK. Moreover, the chronic energy depletion response, which requires TSC1/2, is also found to be AMPK-independent. The importance of these findings are underscored by the observation that in two distinct pre-clinical models of cancer and diabetes, metformin acts to suppress mTORC1 signaling in a TSC1/2- and AMPK-independent manner. The significance of these findings is highlighted by recent record-linked studies showing that metformin is efficacious in the treatment of cancer, as well as type 2 diabetes.

Shikha Khatri

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S6K1 and FOXO3a coordinate survival and metabolism downstream of Akt

Introduction/Rationale: Mutations in the TSC1 and TSC2 genes are thought to trigger the development of LAM by altering the cellular regulation of apoptosis and metabolism. Similar to the TSC genes, the FOXO family of transcription factors is also thought to coordinately regulate cellular metabolism and apoptosis. We investigated whether FOXO3a and S6K1 are cooperative or independent regulators of cellular metabolism and apoptosis.

Methods/Results: Using cell-based assays of metabolism and apoptosis, our results showed that S6K1 is required for Akt-induced glycolysis and suppression of apoptotic cytochrome c release. Interestingly, FOXO3a knockdown correlated with decreased expression of TSC1 and increased S6K1 activation. Rapamycin suppressed increased glycolysis in FOXO3a-deficient cells suggesting a regulatory role for FOXO3a upstream of S6K1. Together the data reveal a novel link between FOXO3a and S6K1 that coordinates glycolysis and apoptosis, and suggest that FOXO3a inactivation could contribute to disease severity in LAM.

Po-Shun Lee, MD
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The Wnt signaling pathway is activated in TSC2-deficient LAM-related cells and tissues, independent of mTORC1

Introduction

Perturbation in Wnt-related pathways has been associated with various human diseases, most notably cancer. Previous studies have identified aberrant beta-catenin signaling in tuberous sclerosis, with enhanced beta-catenin levels in angiomyolipomas and LAM (Mak et al., AJP 05). We explored aspects of beta-catenin-GSK activity in a human cell line model of LAM and in LAM lung sections.

Methods

A TSC2-deficient cell line derived from the angiomyolipoma (AML) of a LAM patient and the corresponding TSC2-addback controls were studied. Pharmacologic inhibition of mTORC1 by rapamycin was tested. mTOR and Rheb inhibition by siRNA knockdown were also performed. Levels of protein expression were quantified by immunoblotting and gene expression levels of Wnt-related proteins were from global gene expression profiling using commercial micro array chips (CodeLink). Immunohistochemistry (IHC) was performed on pulmonary LAM tissues.

Results

The TSC2-deficient AML cell line had higher levels of beta-catenin and phospho-GSK3 in comparison to a TSC2-addback derivative. 24hr treatment with rapamycin of the TSC2-deficient cells had no effect on the levels of beta-catenin or phospho-GSK3. RNA knock down of mTOR and Rheb similarly had no effect on beta-catenin levels. Gene expression profiling revealed abnormal expression of Wnt family proteins by the AML cell line that was also unaffected by rapamycin treatment. IHC showed consistent elevated expression of cytoplasmic and nuclear beta-catenin in both epithelioid and spindle-shaped LAM cells in comparison to normal vascular and airway smooth muscle cells in multiple independent LAM samples.

Conclusions

We conclude that the Wnt-beta-catenin pathway is activated in TSC2-deficient LAM cells. In addition, this activation appears to be due to expression of Wnt family signaling proteins in a manner that is independent of mTORC1 activation. To the extent that WNT pathway activation contributes to LAM pathogenesis, treatment with rapamycin may fail to inhibit this aspect of pathogenesis. Inhibition of the Wnt pathway thus may represent a novel therapeutic target in LAM.

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Expression of melanoma associated antigens in tumors of *Tsc2* heterozygote mice

Tsc2 knockout mice serve as a well characterized model of tuberous sclerosis, angiomyolipoma and LAM. Loss of heterozygosity leads to tumor formation in the liver and lungs, and kidney tumors are observed in virtually all mice within 15 months. In humans, loss of functional TSC2 expression underlies the majority of LAM cases. A diverse array of melanoma-associated antigens is expressed in lung as well as renal lesions, including gp100 recognized by diagnostic antibody HMB45. Mouse models can be important tools to address the opportunity of targeting LAM lesions by immunotherapy. This prompted the current studies to assess expression of melanoma associated antigens and immune infiltration in tumor lesions of *Tsc2* heterozygote mice. Paraffin blocks as well as frozen and fresh tissue from ageing (up to 28 weeks of age) and, in part, ENU treated mice were subjected to immunostaining and FACS analysis of gp100 and GD3 expression. Expression of CD3, CD8, CD11c, CD68 was also analyzed by a combination of immunohistochemistry and FACS analysis. Expression of gp100 was confirmed in lung and liver tissue, and most prominently in kidney tumors of *Tsc2* knockout mice. Loss of tuberin was confirmed in these tumors by comparison to similar tumors observed in *Tsc1* heterozygotes and gp100 expression perfectly colocalized with tuberin tumors. Interestingly, fresh kidney tissue homogenate contained a high percentage of cytotoxic T cells (9% of single cells by FACS analysis). Only 1% of cells were identified as CD11c+ dendritic cells whereas CD68+ macrophages were more prominent (8%). Analysis of tumor-containing lung tissue from a *Tsc2* heterozygote mouse compared to lung tissue from a *Tsc1* heterozygote without obvious tumor formation revealed 15% of cells expressing humoral target molecule GD3 only in tumor containing tissue. The data strongly suggest that *Tsc2* heterozygote mice develop tumors which may be targeted by immunotherapeutic measures targeting gp100 or GD3 to boost anti-tumor responses in a prophylactic or therapeutic setting.

Annabelle Mery
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Investigation of the role of LKB1, AMPK, and the TSC genes in skin tumorigenesis

The LKB1 tumor suppressor is mutated in the familial cancer disorder Peutz-Jeghers Syndrome which shares some common clinical features with Tuberous Sclerosis and Cowden's disease, two other inherited phakomatosis syndromes. The molecular basis for some of the clinical similarities in these diseases has become clear with the discovery that the LKB1 gene is a serine/threonine kinase that directly activates the AMP-activated protein kinase, which directly activates the TSC1-TSC2 complex to inhibit the mammalian-target-of-rapamycin (mTOR) pathway. The LKB1, AMPK, TSC, and mTOR genes have been shown to play key roles in the control of cell growth, cell metabolism, and cell polarity. We examined the phenotypic overlap and requirement for the LKB1, AMPK, and TSC genes to restrain tumor formation within a specific lineage. To that end, we examined the function of these genes in the skin epithelium of mice using tissue-specific inactivation in genetically engineered mice. We find that loss of LKB1 leads to spontaneously arising squamous cell carcinomas, similar to a previous study, which is greatly accelerated by treatment with the skin tumor carcinogenic initiator compound DMBA. Interestingly, loss of TSC in skin epithelium also leads to the formation of squamous tumors though with distinct timing and morphological features. Analysis of the pathways deregulated in LKB1⁻, AMPK⁻, and TSC-deficient skin epithelium will be presented.

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Tasha A. Morrison, PhD
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Estrogen enhances resistance to anoikis in tuberin-deficient cells

Introduction

Genetic evidence indicates that lymphangiomyomatosis (LAM) is the result of benign metastasis of tuberin (Tsc2)-null cells. The molecular mechanisms underlying LAM pathogenesis is not well known. Metastatic cancer cells have the ability to resist anoikis—apoptosis due to loss of extracellular matrix attachment. In a xenograft model of LAM, we found that 17- β -estradiol (E_2) causes a significant increase in circulating tumor cells and promotes lung metastasis. The metastatic phenotypes are associated with the activation of p42/44 MAPK and are inhibited by MEK1/2 inhibitor, CI-1040. We hypothesize that E_2 promotes survival of Tsc2-null cells placed in circulation. To determine the components that mediate estrogen-enhanced survival of Tsc2-null cells, we analyze the pro-apoptotic protein Bim (Bcl-2 interacting mediator of cell death), a critical activator of anoikis. Bim is phosphorylated by p42/44 MAPK, leading to proteasomal-mediated degradation.

Method

ELT3 cells (Eker rat uterine leiomyoma-derived smooth muscle cells) were cultured with or without 10 nM E_2 in serum-free and phenol-red free medium supplemented with 10% charcoal-stripped FBS for 24 hours. Cells were harvested, and plated onto PolyHEMA dishes. Bim and cleaved caspase-3 protein levels were determined by immunoblot analysis. To further understand how Bim is regulated in Tsc2-null cells, cells were treated with proteasome inhibitor, MG132, and MEK1/2 inhibitor, PD98059. For adherent conditions, ELT3 cells were cultured with or without 10 nM E_2 and/or 0.5 μ M MG132 or 50 μ M PD98059 in the same medium. DNA fragmentation was detected using Cell Death Detection ELISA kit.

Results

We found that estrogen decreases levels of cleaved caspase-3 and DNA fragmentation, indicating that E_2 promotes resistance to anoikis. We also found that Bim accumulation is reduced at 1 hour of detachment, which is associated with enhanced cell survival. PD98059 treatment blocks E_2 -reduced Bim and increases levels of cleaved caspase-3 and DNA fragmentation, suggesting that E_2 -induced resistance to anoikis is MAPK-dependent. In adherent cells we found that estrogen decreases Bim transcripts at 24 hours of E_2 stimulation, measured by real-time RT-PCR. E_2 also reduces Bim protein levels. Pre-incubation of cells with MG132 and PD98059 blocks estrogen's reduction of Bim. In conclusion, we found that E_2 regulates pro-apoptotic protein Bim, in Tsc2-null cells. Inhibition of MEK1/2 and proteasomal activity block E_2 -regulated Bim accumulation and cell survival. We anticipate that targeting signaling pathways contributing to Bim activation and proteasome activity may have clinical specificity and significance in treating LAM.

Tiffany Whitney, PhD
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LAM-derived cells Express a malignant and lung metastatic expression signature (LMS)

Although LAM lesions have historically been viewed as benign, recent reports suggest that LAM cells exhibit metastatic and neoplastic potential. We constructed a LAM-derived SAGE library and found significantly altered expression of hundreds of genes. We focused on a subset of those genes known to be involved in angiogenesis, proliferation, tissues remodeling, metastasis and immune evasion with special emphasis on a small fraction that are also associated with increased lung metastatic potential.

Despite the fact that most tumors are made up of a heterogeneous population of cells, specific expression patterns confer primary tumors with enhanced metastatic potential resulting in poor patient prognosis. Certain expression signatures have even been associated with tissue specific metastatic potential. Our data reveal that LAM-derived cells express a signature that is associated with enhanced metastasis to the lung. These cells exhibit significantly increased transcription of *MMP1*, *MMP2*, *PTGS2/Cox2*, *SPARC*, *CDK1*, *TNC*, *CXCL1* and *UGT8*. The combined upregulation of these genes is associated with augmented metastasis to the lung and has been termed a “lung metastasis gene expression signature (LMS)”. This signature not only confers increased metastatic potential, but specifically enhances the potential of cells to metastasize to, colonize and survive within the lung. Increased *STAT3* activity is seen in many cancers and is often associated with more aggressive tumors. Increased tyrosine phosphorylation at position 705 of *STAT3* has been documented in LAM lesions and our library reveals that many of the downstream targets involved in angiogenesis, proliferation and immune evasion show altered expression indicative of constitutively active *STAT3*. *STAT3* decreases the immune response in part by inhibiting expression of *TNF*, *CCL5* and MHC Class II molecules. We see a significant reduction or complete absence of these transcripts in our library. Accordingly, *STAT3* induces angiogenesis by upregulating *bFGFs*, *HGF* and *MMP2* which all show significantly increased expression in our library.

In addition to the aberrant TGF-beta signaling which we previously reported, we also observed increased transcription of TGF-beta-induced (beta-ig-h3) transcript, which is directly involved in extravasation of cells from blood and lymph vessels during metastasis. Beta-ig-h3 is also known to induce *SRC* activation, which in turn increase *STAT3* activity.

Our data show that LAM-derived cells express a molecular signature commonly found in malignant cells and suggest that a complex network of signaling pathways and molecular mechanisms are involved. Because the pulmonary manifestations of LAM result in patient morbidity and mortality, it is important to understand the mechanism by which LAM cells are able to specifically target, invade and thrive within the lung. These data reveal possible therapeutic targets that may prevent metastasis of LAM cells to the lung.