Analysis of the oestrogen response in an angiomyolipoma derived xenograft model

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Abstract

Angiomyolipomas are benign mesenchymal tumours of smooth muscle, blood vessels and fat which occur sporadically or associated with tuberous sclerosis and lymphangioleiomyomatosis (LAM), a rare cystic lung disease. Angiomyolipoma and LAM are caused by loss of function of either the tuberous sclerosis-1 or -2 genes resulting in activation of p70S6kinase (S6K1) and uncontrolled cellular proliferation. LAM and angiomyolipoma can be exacerbated by oestrogens but how this occurs is not understood. To address this question, we created a xenograft tumour system in nude mice using immortalised angiomyolipoma cells. Angiomyolipoma xenografts had active S6K1, p38, p42/44 MAPK and Akt; they grew more rapidly and had greater Akt phosphorylation after oestrogen treatment of tumour-bearing mice. Transcriptional profiling showed oestrogen induced 300 genes including extracellular matrix proteins, proteases, cell cycle regulatory proteins and growth factors including platelet derived growth factor-C (PDGF-C). Biologically active PDGF-C was produced by primary angiomyolipoma cells in culture and PDGF-C protein was present in the neoplastic smooth muscle cells of 5/5 human angiomyolipoma and 4/5 LAM tissues examined by immunohistochemistry. These findings suggest that the response to oestrogen in this model is mediated by activation of Akt and transcriptional events. This model may prove useful for studying the biology and effect of drugs on angiomyolipoma and diseases related to TSC.

Endocrine-Related Cancer (2009) 16 59–72

Introduction

Angiomyolipomas are mesenchymal neoplasms composed of adipose tissue, blood vessels and abnormal smooth muscle cells, the latter generally in a perivascular pattern. Angiomyolipomas most commonly occur in the kidneys and, although often asymptomatic, may enlarge and bleed leading to haemorrhage and renal impairment (Ewalt *et al.* 1998, Bissler & Kingswood 2004). The lesions are usually benign although rare malignant cases have been described, often consisting of only the smooth muscle component (Hornick & Fletcher 2006). The smooth muscle cell component of angiomyolipoma is thought to derive from a precursor termed the perivascular epithelioid cell (PEC; Pea *et al.* 1997). PEC-derived lesions, which includes lymphangioleiomyomatosis

(LAM), a cystic lung disease, are characterised by the dual expression of smooth muscle (including α -smooth muscle actin) and melanocytic features (including melanoma antigen recognised by T-cells (MART-1) and HMB-45 (L'Hostis *et al.* 1999)) at both an immunohistochemical and ultrastructural level; a normal tissue counterpart of these cells has not been identified.

Angiomyolipoma is rare in the general population but is present in 80% of patients with tuberous sclerosis (TSC), an autosomal dominant tumour suppressor syndrome characterised by multiple hamartomas in the central nervous system, skin and other organs (Casper *et al.* 2002). Angiomyolipoma, LAM and TSC share a genetic aetiology in that loss of function of one out of the two TSC genes, TSC-1 or more commonly TSC-2, is seen in all three entities. The TSC-1 and -2

proteins, termed hamartin and tuberlin respectively form a complex that inhibits the mammalian target of rapamycin (mTOR)/raptor containing complex (TORC1), a pivotal cellular kinase governing proliferation and apoptosis (Li *et al.* 2004).

Although TSC occurs equally in men and women, angiomyolipoma is more common and larger in women and is thought to be oestrogen dependent. LAM also occurs almost exclusively in women (Franz *et al.* 2001). LAM and angiomyolipoma cells have oestrogen receptor α (Logginidou *et al.* 2000, Matsui *et al.* 2000), and exogenous oestrogen is thought to cause clinical worsening in women with LAM (Johnson & Tattersfield 2000). *In vitro*, the growth of cells derived from angiomyolipoma can be enhanced by oestrogen (Yu *et al.* 2004). Thus, it is likely that although LAM and angiomyolipoma result from the loss of tuberlin or hamartin, unlike other TSC related hamartomas, and their growth is promoted by oestrogen. There is some evidence that oestrogen sensitivity may be dependent upon activation of mTOR in TSC derived cells suggesting an interaction between the oestrogen and mTOR pathways (Finlay *et al.* 2004). Despite this, little is known of how oestrogen causes LAM and angiomyolipoma progression. To address these questions, we set out to establish a model system that was oestrogen responsive and could be used to study the biology of the disease. Using an immortalised angiomyolipoma cell line developed by Arbiser *et al.* (2001), we have generated an oestrogen responsive mouse xenograft model to examine how oestrogen leads to increased growth in angiomyolipoma and LAM.

Methods

Angiomyolipoma cells, tissue and ethical approvals

Human angiomyolipomas from patients with LAM and TSC have been collected from excess material obtained for clinical purposes from patients on the UK LAM database. LAM and angiomyolipoma were confirmed by standard histological assessment by the reporting pathologist including specific immunostaining for α -smooth muscle actin, HMB-45 and in some cases oestrogen and progesterone receptors. Ethical approval for the UK LAM database has been obtained from the Trent Multi Centre Research Ethics Committee and the use of angiomyolipoma tissue and primary angiomyolipoma cell culture from Nottingham Research Ethics Committee; informed consent was obtained. SV7^{tert}AML cells were obtained from ATCC

(ATCC CRL-2461) and cultured in DMEM with 10% foetal bovine serum (FBS). Primary cells were cultured from angiomyolipoma resections performed for therapeutic purposes. Excess material not required for clinical use was divided into small ($\sim 3 \times 3 \times 3$ mm) fragments, treated with type II collagenase and the resulting cell suspensions plated into T25 tissue culture flasks in LAM medium (adapted from reference Goncharova *et al.* 2002) comprising phenol red-free DME/F12 medium, plus ferrous sulphate 1.6×10^{-6} M, vasopressin 1.2×10^{-5} units/ml, triiodothyronine 1×10^{-9} M, insulin 0.025 mg/ml, cholesterol 1×10^{-8} M, transferrin 10 pg/ml supplemented with 15% FCS and 5 ng/ml epidermal growth factor. Most flasks yielded only cells with spindle shaped morphology, which proliferated relatively slowly. A small number of flasks also contained highly proliferative cells of epithelioid morphology: these cells rapidly outgrew these cultures such that after two passages, they were the only cell type evident on inspection. This distinction in morphology was also reflected in differences in gene expression as determined by RT-PCR. Human airway smooth muscle (ASM) cells were grown from explants from the large airways of surgical resection specimens. Ethical permission has been received from the Nottingham Research Ethics Committee. These cells were grown in DMEM with 10% FBS at 37 °C and 5% CO₂, and were used between passages 4–6.

Xenograft model and animal care

In vivo studies were performed under UKCCCR guidelines (UK, Home Office Project Licence number 40/2323). Nude mice were bred on site from animals originally obtained from Harlan UK Ltd, and were housed in pathoflex isolators at 26 °C, on 12h light:12h darkness cycles. Irradiated RB2 diet and autoclaved water provided *ad libitum*. Xenografts were established by s.c. injection of 2×10^6 SV7^{tert}AML cells in 100 μ l PBS into the left flank of female nude mice in the presence or absence of oestrogen. Oestrogen was supplied as 0.36 mg 17 β -oestradiol 60-day release pellets placed subcutaneously (Innovative Research of America); these pellets also contained a matrix comprising cholesterol, cellulose, lactose, phosphates and stearates. This dose of oestrogen gives physiological replacement levels (Babiker *et al.* 2007) and is regularly used in xenograft models in our unit.

Resulting tumours were excised from donor animals, finely minced and 3 mm³ pieces subcutaneously implanted into the left flank of juvenile female nude mice under anaesthetic (Hypnorm,

Roche/Hypnovel, Jansen), a method resulting in reproducible non-ulcerating tumours in earlier studies. Mice were weighed weekly and their clinical condition monitored by a trained observer. Tumour size was measured three times weekly using callipers in two perpendicular dimensions. Mice were terminated individually when the tumour cross-sectional area reached 250 mm² or sooner, which has previously been seen to be below the limit of 10% of initial body weight set by the UKCCCR guidelines. Tumours were excised, weighed and portion immediately snap frozen in liquid nitrogen and stored at 70 °C, for protein and mRNA extraction, the remainder was preserved for immunohistochemical staining.

PCR

For standard reverse transcriptase PCR (RT-PCR) cells were grown to confluence in 75 cm² tissue culture flasks and RNA extracted using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen Ltd). Contaminating DNA was removed using DNase I (Qiagen) and cDNA synthesised using Superscript II Reverse Transcriptase (Invitrogen). RT-PCR was carried out using Invitrogen Recombinant Taq according to the manufacturer's instructions, in a Biometra Thermal Cycler. PCR cycling conditions were: 94 °C × 30 s, 60 °C × 30 s, and 72 °C × 30 s for 35 cycles. Products were resolved on 2% agarose gels and visualised by ethidium bromide staining. For real-time, quantitative RT PCR, cDNA samples were initially amplified using Invitrogen recombinant Taq in a Biometra Thermal Cycler, and products analyzed on a 2% agarose gel stained with ethidium bromide, to ensure that a single band of the appropriate length was amplified. Relative quantification of mRNA was performed using a SYBR Green quantitative PCR method using *GAPDH* and *β-actin* as normalisation controls. All reactions were performed in triplicate on an Applied Biosystems 7500 Real Time PCR System using Eurogentec qPCR Core Kit for SYBR Green I. The reaction contained: 2.5 µl 10 × RB, 1.75 µl 50 mM MgCl₂, 1 µl 5 mM dNTPs, 0.75 µl SYBR Green, 0.13 µl HotStar Taq, 0.25 µl UNG and 1 µl of 10 µM primer mix, in addition to cDNA, in a reaction volume of 25 µl. Validation of primer efficiency was performed by dilution curve construction. Melt curve analysis was performed on the reaction products to ensure that a single amplification peak between 80–90 °C was obtained. The reaction was carried out for 40 cycles with the following conditions: 95 °C × 30 s; annealing at 60 °C × 30 s; and extension at 72 °C × 30 s. Each PCR generated only the expected

amplicon as shown by the melting temperature profiles of the final products. Primers were used as follows: *gp100* F, R (forward, reverse), TGGCTCTTGGT-CTCAGAAGA, AGGTGCAGTGCTTATGACTT. *MART1* F, R AGATGCCAAGAGAAGATCTC, GCTCTTAAGGTGAATAAGGTGG. *PDGFC* F, R CTTGAACCAGAGAGATGGCAG, GGACACTGA-GAAGTTACGAGG. *PDGFA* F, R ACACGAG-CAGTGTC AAGTGC; CCTGCAGTATTCCACCTT-GG *PDGFB* F, R AGATCGAGATTGTGCGGAAG; CAGCTGCCACTGTCTCACAC *PDGFD* F, R CCC-AGGAATTACTCGGTCAA; ACAGCCACAATTT-CCTCCAC *PDGFRA* F, R TGGGAGTTTCCAAGA-GATGG; TGTTCCTTACCACCTTCC; *β-actin* F, R CCTGGCACCCAGCACAAAT, GGGCCGGACTC-GTCATACT; *GAPDH* F, R CTCTCTGCTCCTCCT-GTTCGAC, TGAGCGATGTGGCTCGGCT.

Immunohistochemistry

Mouse xenograft paraffin sections were dewaxed in Histoclear (RA Lamb Ltd, Eastbourne, UK), rehydrated through an ethanol series, subjected to antigen retrieval by boiling in 10 mM citrate buffer for 10 min, and incubated with primary antibodies detailed below, using an Animal Research Kit (Dako, Ely, Cambridge-shire, UK) according to the manufacturers instructions, using 3,3-diaminobenzidine (DAB) as the chromogen and counterstained with haemalum. Antibodies used were against α -smooth muscle actin (Sigma), oestrogen receptor α clone 1D5 (Dako), oestrogen receptor β (rabbit polyclonal, Lab Vision, Fremont, CA, USA); phospho p70S6kinase^(T389) (Abcam, Cambridge, UK) and HMB-45 (Serotec, Kidlington, Oxford, UK). Human paraffin embedded sections were immunostained using antibodies detailed below and the Vectastain Elite ABC kit according to the manufacturer's instructions (Vector Laboratories, Peterborough, UK) using DAB as the chromogen and haemalum as a counterstain. Antibodies used were goat anti-human PDGF-C (R&D systems Abingdon, Oxford, UK) at 2 µg/ml, and mouse anti α -smooth muscle actin (Sigma Aldrich). Negative controls were matched normal IgG as appropriate and omission of the primary antibody. Immunostaining was examined by an experienced pathologist (TAM) and scored visually as absent, positive or strongly positive.

Western blotting

Tumour lysates were obtained from xenografts, and equal quantities of tissue were homogenised by sonication. Homogenates were resuspended in Lysis Buffer (50 mM Tris–Cl (pH 7.5), 150 mM NaCl, 1 mM

EDTA, 1% NP-40), including protease and phosphatase inhibitors, for 15 min on ice and insoluble debris removed by centrifugation at 10 000 g for 10 min. For preparation of lysates from adherent cells in culture, culture medium was aspirated, the cells rinsed briefly in with PBS, and then SDS-loading dye was added directly to the cells. Proteins were resolved by electrophoresis on 10% or 7.5% SDS-polyacrylamide gels, then blotted onto Hybond-P membrane (Amersham Biosciences) and probed with anti-total and anti-phospho Akt (Ser473), p42/44 MAPK, p38 MAPK, p70S6kinase (Thr389; Cell Signalling Technologies, Danvers, MA, USA), tubulin clone BS 2-1 (Sigma Aldrich). Secondary antibodies were HRP-conjugated goat anti-rabbit IgG and HRP-conjugated goat anti-mouse IgG (Sigma Aldrich). Signals were visualised using the ECL Western Blotting Detection Kit (Amersham Biosciences). Bands from 11 tumours were checked for equal protein loading using β -tubulin and each group was quantified by densitometry, expressed as percent of control values and compared using an unpaired *t*-test with 5% taken as significant.

Bromodeoxyuridine uptake

To study cellular proliferation within the tumours mice were injected with 50 mg/kg bromodeoxyuridine (BrdU) 1 h prior to termination. BrdU incorporation was quantified in paraffin sections from xenograft tumours by immunohistochemistry using a biotinylated primary anti-BrdU antibody and streptavidin conjugated peroxidase (Dako), detected using DAB. The percentage of positive staining cells in five tumours from each group was calculated in ten low power fields from each tumour.

Transcriptional profiling and analysis

Tumours were removed and a portion flash frozen in liquid nitrogen. RNA was extracted from five tumours from oestrogen-treated and control animals. Equal quantities of RNA were pooled and quality checked by Agilent Bioanalyser. Transcriptional profiling was performed at the Nottingham Arabidopsis Stock Centre (Craigon *et al.* 2004). Twenty micrograms of RNA from each group was used to produce biotin-labelled complementary RNA that was hybridised to an Affymetrix Human Genome U133+2 set. Gene expression data were analysed using GeneSpring (Silicon Genetics, Agilent Technologies, West Lothian, UK). Data were normalised and filtered to express genes, which were induced or repressed by twofold or greater. The dataset was submitted to the NCBI Gene Expression Omnibus (Series record GSE5868).

Growth and proliferation assays

For thymidine incorporation assay ASM cells were grown in 24-well plates until 90% confluent then serum depleted for 24 h. Two hundred microlitre of primary AML culture conditioned or identical non-conditioned medium was added to culture medium in a 1:3 ratio with DMEM. In some experiments, 20 μ g/ml anti-PDGF-C blocking antibody (R&D systems) or isotype matched control were added to primary AML conditioned media. These experiments were conducted in the presence of serum as the high concentrations of immunoglobulin required to achieve blocking activity (ND_{50} for thymidine incorporation against 0.8 μ g/ml PDGF-C was 6–24 μ g/ml: manufacturer's data) had non-specific effects on the ASM cells that affected the proliferation assay in the absence of serum. Tritiated thymidine (1 μ Ci/well) was added for 16 h. At 24 h, the supernatant was removed and DNA precipitated by addition of 10% trichloroacetic acid at 4 °C for 30 min. The TCA was aspirated and cells lysed in 0.2 M sodium hydroxide solution at 4 °C overnight. The lysate was then added to 10 ml scintillation fluid and disintegrations per minute quantitated by scintillation counting. For each experiment, fresh conditioned medium was obtained and a separate primary ASM cell line used. As the response of individual primary cultures varied, experiments were compared as fold mean increase over baseline. For growth of primary angiomyolipoma and SV7^{tert}AML cells we used an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. After incubation with growth factors or oestrogen, 1/10th volume of 5 mg/ml MTT was added to wells and incubated at 37 °C for 1 h, the medium was removed, 200 μ l MTT solubilisation solution added (Cell Proliferation Assay Kit, Sigma), the plates shaken for 5 min and optical density read at 570 nm. In both assays, experimental conditions were run in at least triplicate wells with at least three independent experiments performed. Data were compared using an unpaired *t*-test with 5% taken as significant.

Reagents

LY294002 and wortmannin were obtained from Calbiochem (Nottingham, UK) Rapamycin was obtained from AG Scientific (Derbyshire, UK). Tissue culture plastic was obtained from Corning (Flintshire, UK). All other reagents and chemicals were obtained from Sigma unless otherwise stated.

Results

Generation of AML xenograft tumours

To confirm dysregulation of the mTORC1 pathway in SV7^{tert} AML cells, western blotting was used to demonstrate constitutively phosphorylated p70S6K1 at threonine 389 in the absence of serum; this phosphorylation was completely abolished by the mTORC1 inhibitor rapamycin (100 nMol for 3 h) but not the PI3 kinase inhibitor wortmannin. Primary human ASM cells were also included as a wild type control, and display a very low level activation of p70S6K in the absence of serum. Akt and p42/44 MAPK were not constitutively active in the absence of serum in either cell type (Fig. 1a).

Two million SV7^{tert} AML or primary human angiomyolipoma cells were subcutaneously injected into nude mice either with or without s.c. oestrogen pellets ($n=4$ per group). No tumour resulted in the primary angiomyolipoma groups after 6 months. Similarly, no tumour formed in the non-oestrogen-treated SV7^{tert} AML mice. However, all oestrogen-treated mice inoculated with SV7^{tert} AML cells developed s.c. tumours, which required termination of the animals at 6 weeks due to tumour load. These primary tumours were removed and fragments placed subcutaneously in further nude mice, which went on to develop tumours (termed angiomyolipoma xenografts) both in the presence and absence of oestrogen.

Angiomyolipoma xenografts were excised and processed for immunohistochemistry. The tumours had a malignant epithelioid appearance with marked nuclear pleomorphism, no vascular cell or adipocyte was present. Immunohistochemistry of xenografts was positive for α -smooth muscle actin, oestrogen receptor (ER)- β , and phosphorylated p70S6K1 (Fig. 1b). There was no reaction for HMB-45 (Fig. 1b) or oestrogen receptor (ER)- α (not shown) by immunohistochemistry.

AML xenograft growth is oestrogen sensitive

As tumour formation appeared oestrogen dependent this was studied formally. Twelve nude mice received s.c. angiomyolipoma xenografts, six of which were also implanted with 60-day slow release s.c. oestrogen pellets. Angiomyolipoma xenografts grew in both control and oestrogen-treated mice but growth was significantly faster in oestrogen-treated animals (Fig. 2a) with final tumour weights being 0.14 g (± 0.07 s.d.) in the control groups and 0.36 g (± 0.16) in the oestrogen-treated group ($P=0.02$, $n=6$). In a second identical experiment, mice were labelled with BrdU to evaluate DNA synthesis.

Figure 1 Characteristics of angiomyolipoma xenografts. (a) Western immunoblot of SV7^{tert} AML and human airway smooth muscle (HASM) lysates for S6K1 phosphorylated at threonine 389 (p-S6K1), phospho-AKT (Ser473) and phospho-p42/44 MAPK in the presence and absence of 10% serum, the mTORC1 inhibitor rapamycin and the PI3kinase inhibitor wortmannin. Cells were serum deprived for 72 h, then treated with/without 10% foetal bovine serum for 3 h in the presence or absence of inhibitors. S6K1 phosphorylation is observed in SV7 Tert cells grown in the absence of serum. This activation is eliminated by treatment with 100 ng/ml rapamycin for 3 h, but not by 100 nM wortmannin. Treatment with 10% serum for 3 h elicits a robust activation of phospho-p70S6K in both cell types, which is sensitive to rapamycin but relatively insensitive to wortmannin. Blots were probed for total S6K1, AKT and p42/44 as loading controls. (b) Haematoxylin and eosin (H&E) and immunohistochemical staining (3,3'-diaminobenzidine (DAB) chromogen counterstained with haematoxylin) of mouse xenograft tumours showing positive immunostaining for phospho-S6K1, oestrogen receptor β and α -smooth muscle actin but not HMB-45 (Magnification 400 $\times$, HMB-45 magnification 100 $\times$).

Figure 2 Angiomyolipoma xenograft growth is oestrogen dependent. (a) Growth curves for AML xenografts in control and oestrogen-treated mice, shown as mean ($\pm$ s.e.m.) tumour cross sectional area (CSA) against days since tumour implant for six mice per group. (b) Mean ($\pm$ s.e.m.) DNA incorporation in tumours ($n=5$ per group) as judged by BrdU incorporation was not significantly different between groups.

Tumour growth was again faster in the oestrogen-treated group but there was no significant difference in BrdU incorporation between control and oestrogen-treated animals (Fig. 2b).

Akt and MAPK pathways are activated in AML xenografts

We examined the activation of Akt, p38 MAPK and p42/44 MAPK in angiomyolipoma xenografts. Tumours were extracted from nine oestrogen-treated and nine control mice, cellular proteins were isolated and equal protein concentrations were examined by western blotting. All three signalling intermediates were active as judged by the presence of the phosphorylated proteins. Further, a 60% increase in Akt^{Ser473} phosphorylation was seen in the tumours of oestrogen-treated animals although total Akt, total and

phosphorylated p38 MAPK and p42/44 MAPK were not affected by oestrogen treatment (Fig. 3).

Transcriptional profiling of oestrogen-dependent genes

To address which genes were associated with oestrogen-dependent growth of these tumours we used a transcriptional profiling approach. RNA was extracted from five tumours from oestrogen-treated and five from control mice and was pooled for each group. Labelled anti-sense RNA probe preparation and hybridisation to an Affymetrix Human Genome

Figure 3 p42/44 MAPK, p38 MAPK and Akt signalling in angiomyolipoma xenografts. Graphs show mean densitometry of western blots normalised to control expression levels from oestrogen-treated and control mice ($n=9$, $\pm$ s.e.m./group) for total and phosphorylated p42/44 MAPK, p38 MAPK and Akt. Phospho-Akt^{Ser473} is increased by 60% ($\pm$ 11%) by oestrogen treatment.

U133+2 chip set was carried out. Three hundred genes were induced and 356 repressed by twofold or greater in oestrogen-treated tumours. Table 1 shows known genes up-regulated by oestrogen. These include a significant number of extracellular matrix proteins

associated with tumour growth including fibronectin (and its receptor β 1-integrin), laminin- β 1, collagens, matrix degrading proteases including ADAM 9, 10, ADAMTS 2 and 7. Strongly up-regulated were tumour associated protein 52, tumour associated Ca^{2+} signal

Table 1 Genes induced twofold or greater over control values in oestrogen-treated angiomyolipoma xenografts (fold induction over control)

Extra cellular matrix	Tumour related
Collagen IV (6.2)	Tumour protein D52 (6.5)
Collagen VIII (4.0)	Tumour associated Ca^{2+} signal transducer (4.7)
Fibronectin I (2.9)	BRCA1 interacting protein C-terminal helicase (2.8)
Microfibrillar acidic protein 3 (2.9)	CD9 antigen (2.7)
Fibrillin II (2.7)	Jun D (2.2)
Collagen XII 1 α (2.4)	Transcription factors
Laminin- β 1 (2.4)	NGF1A-binding protein (3.7)
Elastin microfibril interfacier (2.3)	TAF9-like RNA polymerase II TBP associated factor (3.1)
Fibulin I (2.3)	Replication factor C (3.0)
Nidogen 2 (2.2)	Runt related transcription factor 3 (2.9)
Proteases	Transcription factor 8 (2.8)
Cathepsin Z (3.6)	Matrin 3 (2.5)
ADAM 10 (3.2)	CCAAT binding transcription factor (2.2)
Pappalysin 1 (2.6)	Sp 1 (2.1)
Calpain 6 (2.5)	Transporters
Ubiquitin-specific protease 34 (2.4)	CDW92 (4.0)
ADAM 9 (2.3)	Importin α 3 (2.3)
ADAMTS 2 (2.2)	Cell cycle
ADAMTS 7 (2.1)	Cyclin G2 (3.0)
Growth factors (GF)/GF related	CDK2 (2.4)
IGFBP 2 (3.5)	P21 activated kinase 2 (2.2)
PDGF C (3.1)	Apoptosis
FGF2 (2.1)	MCL1 (2.8)
TGF α (2.1)	Fas-associated factor 1 (2.2)
Neuregulin 2 (2.0)	Other
Smooth muscle proteins	Sphingosine 1 phosphate phosphatase (4.7)
Actin γ 2 (3.0)	Thymopoietin (4.2)
Troponin C (2.6)	Lymphocyte antigen 75 (3.8)
Tropomyosin (2.1)	Semaphorin 3c (3.7)
Tropomodulin 3 (2.1)	Glutamine synthase (3.3)
Actin β (2.1)	Protein tyrosine phosphatase non-receptor 11 (3.3)
Caldesmon I (2.1)	UDP-GlcNAc: β Gal β -1,3- <i>N</i> acetylglucosaminyltransferase 1 (3.2)
Signal transduction	Squalene epoxidase (3.2)
G protein α 13 (3.3)	Catenin (2.9)
PKC ϕ (2.5)	Inhibitor of DNA binding 4 (2.9)
PKC α (2.1)	ADP ribosylation factor-GEF 1 (2.7)
JAK 1 (2.1)	GPI dacylase (2.6)
SMAD 4 (2.1)	Splicing factor 2 (2.6)
Ca^{2+} activated nucleotidase 1 (2.1)	Lysosomal-associated protein transmembrane 4 β (2.5)
MAPKKK (2.0)	TRPC1 (2.5)
Signal transduction modulators	Calnexin (2.5)
Tissue specific extinguisher 1 (2.5)	Mannosidase α (2.5)
Chordin (2.2)	Chloride intracellular channel 4 (2.5)
FK506-BP (2.0)	Histone deacetylase 4 (2.3)
Surface receptors	Carboxypeptidase D (2.3)
Integrin β 1 (2.6)	Ethanolamine kinase (2.3)
BMPT 2 (2.5)	Heat shock protein 1 (2.3)
Sortilin 1 (2.5)	Inhibin β A (2.3)
Chemokine orphan receptor 1 (2.3)	Formin 2 (2.1)
IL 13 R (2.2)	Ezrin (2.0)

transducer and BRCA1 interacting protein C-terminal helicase. Cell cycle associated proteins, smooth muscle structural proteins and various signal transduction proteins were also up-regulated (Fig. 4). The full dataset is available online at <http://www.ncbi.nlm.nih.gov/projects/geo> (Series record GSE5868). The growth factor PDGF-C was induced 3.1 fold by oestrogen. We studied PDGF-C further as it is associated with growth, angiogenesis and lymphangiogenesis, potentially important aspects of oestrogen-dependent growth of angiomyolipomas and LAM. Up-regulation of PDGF-C mRNA in individual AML xenografts was independently confirmed using real-time RT-PCR. Treatment with oestrogen resulted in a 1.9 fold increase over control animals in PDGF-C mRNA ($n=3$ per group, $P=0.047$).

Expression of PDGF-C in human angiomyolipoma and LAM

To examine the presence of PDGF-C in angiomyolipoma and the related disease LAM, PDGF-C was examined by immunohistochemistry in paraffin embedded tissues obtained from resection, biopsy and lung transplant procedures. Five angiomyolipomas from separate patients were stained for PDGF-C, all were positive in normal renal tubules and vascular smooth muscle as expected (Fig. 5; Uutela et al. 2001) and, in the neoplastic smooth muscle derived cells although staining intensity varied between samples. The smooth muscle component of angiomyolipoma and LAM cells stained strongly for α -smooth muscle actin. Five cases of LAM were examined for PDGF-C, unlike angiomyolipoma PDGF-C was expressed to a

variable degree in LAM cells from four cases and absent in one (Fig. 5).

Characterisation of primary angiomyolipoma cells and expression of PDGF-C

To confirm the expression of PDGF-C in primary angiomyolipoma cells we studied cells derived from a TSC-related angiomyolipoma. Consistent with previous studies (Lesma et al. 2005), this angiomyolipoma produced two populations of cells which had either a spindle shaped or epithelioid appearance; the epithelioid cells were most similar morphologically to SV7^{tert}AML cells (Fig. 6a; Lesma et al. 2005) and also had constitutively active S6K1 (not shown). Both cell types expressed α -smooth muscle actin; spindle shaped cells had prominent stress fibres, whereas epithelioid cells had globular actin staining (Fig. 6a). mRNA for the melanoma related protein GP100 was present in both cell types and *MART1* was present in the spindle cells (Fig. 6b). The cells proliferated in response to oestrogen and PDGF consistent with other reports (Yu et al. 2004; Fig. 6c).

PDGF-C mRNA was detectable in these cells by RT-PCR (Fig. 7a), and was also detected in SV7^{tert}AML cells and ASM cells. Quantitation of transcript levels (relative to β -actin) of PDGF family members revealed that *PDGF-C* was the most abundant transcript. Expression of the PDGF-C receptor, *PDGFR α* , was also detected (Fig. 7b).

We then went on to examine whether oestrogen increased the expression of *PDGF-C* in the primary AML cells. Interestingly, oestrogen had no effect on *PDGF-C* mRNA expression, and in keeping with this,

Figure 4 Oestrogen dependent genes in angiomyolipoma xenografts. Pie chart to illustrate classes of genes induced twofold or greater by oestrogen.

Figure 5 Immunohistochemical staining of angiomyolipoma and LAM tissue. Chromogen DAB counterstained with haematoxylin (magnification 100 $\times$).

oestrogen treatment of primary AML cells did not enhance the effect of conditioned medium on ASM proliferation (not shown).

To assess whether primary angiomyolipoma cell derived PDGF-C is biologically active, we examined the effect of primary angiomyolipoma cell derived conditioned medium on the proliferation of primary human ASM that express PDGF receptor α and proliferate in response to PDGF receptor α agonists (Johnson & Knox 1999). Control, non-conditioned and 24 h-conditioned media from primary angiomyolipoma cells were added to primary ASM cells (1:3 in serum-free media) for 24 h and ASM proliferation assessed by thymidine incorporation. As expected, in low serum conditions (1% FBS), conditioned medium, resulted in a 4.2 fold ($n=4 \pm 0.6$) increase in thymidine incorporation over identical non-conditioned medium.

Figure 6 Characterisation of primary angiomyolipoma cells. (a) Phase contrast microscopy and immunofluorescence for α -smooth muscle actin in spindle shaped and epithelioid primary angiomyolipoma cells. Spindle-shaped cells have well developed stress fibres staining strongly for α -smooth muscle actin whereas α -smooth muscle actin in epithelioid cells is diffuse. (b) RT-PCR for the melanoma markers *MART-1*, *GP100*, in spindle (S) and epithelioid (E) primary angiomyolipoma and SV7^{terc} AML (T). For comparison, cells of melanocytic (Bowes melanoma, B) and non-melanocytic (primary airway smooth muscle cells, A) are included. *GAPDH* is shown to confirm presence of cDNA. (c) Primary angiomyolipoma cell number assessed by MTT cleavage is increased by serum, PDGF and oestrogen.

To examine whether PDGF-C contributed to the mitogenic effect of primary AML cell medium, 20 μ g/ml anti-human PDGF-C blocking antibody or isotype matched control were added to primary AML conditioned media which was then added to ASM cells and proliferation assessed by thymidine incorporation. The PDGF-C blocking antibody caused a 37% ($P=0.01$) reduction in thymidine incorporation over isotype matched control IgG (Fig. 7c). Recombinant human PDGF-C (10–50 ng/ml) also caused proliferation of the SV7^{terc} AML cells (Fig. 7d).

Discussion

We have generated a xenograft system with which to study angiomyolipoma and the related disease LAM. Oestrogen treatment resulted in increased angiomyolipoma xenograft growth. We used three methods to examine the mechanisms underlying the oestrogen response, namely the activity of key signalling pathways as judged by protein phosphorylation, the transcriptional profile in response to oestrogen in our model and the growth response of primary AML cells to oestrogen and other mitogens.

LAM and angiomyolipoma tissue and cells derived from these lesions have constitutively active p70S6kinase (Goncharova *et al.* 2002, El-Hashemite *et al.* 2003). In addition to p70S6kinase, various groups have demonstrated TSC-2 deficient cells, have constitutively active p38 MAPK (Karbowiczek *et al.* 2004), and p42/44 MAPK (Yu *et al.* 2004). In AML xenografts, oestrogen treatment was associated with increased Akt phosphorylation but not p38 MAPK or p42/44 MAPK phosphorylation. Oestrogen-induced Akt phosphorylation has also been observed in breast cancer cell lines (Tsai *et al.* 2001) and recently, tuberlin deficient ELT3 cells (Yu & Henske 2006). Akt, a pivotal cellular kinase has multiple roles in tumourigenesis including growth promotion by inhibition of p53 via nuclear translocation of MDM2, cytoplasmic localisation of p21 and p27, and stabilisation of cyclin D1/D3. Further, Akt also inhibits apoptosis by inactivation of BAD and pro-caspase 9 and inhibition of forkhead transcription factors and hence Fas expression (Testa & Bellacosa 2001). Consistent with these observations at a molecular level, phosphorylation of Akt is associated with a poor prognosis in oestrogen receptor positive breast cancer (Kirkegaard *et al.* 2005). Thus, activation of Akt is a potentially important event in oestrogen-mediated growth of angiomyolipoma xenografts as in other

Figure 7 Expression of PDGF-C in primary AML cells, angiomyolipoma and LAM tissue. (a) RT-PCR shows presence of *PDGF-C* transcript in HASM, SV7^{tert} AML and primary AML cells. (b) Quantitative RT-PCR to show the relative abundance (normalised to β -actin) of PDGF isoform and receptor transcripts in SV7^{tert} AML and primary AML cells. (c) Conditioned medium (CM) from primary angiomyolipoma cells is mitogenic for primary airway smooth muscle cells and treatment with a PDGF-C blocking antibody (anti-PDGF-C, 20 μ g/ml) reduces primary angiomyolipoma conditioned medium stimulated thymidine incorporation. Representative experiment of three shown: FBS, foetal bovine serum; IgG, isotype control immunoglobulin; DPM, disintegrations/minute. (d) PDGF-C (50 ng/ml) causes a 40% increase in MTT reduction in SV7^{tert} AML cells. Graphs show mean ($\pm$ S.E.M.) of three to six independent experiments (* P < 0.05, ** P < 0.01).

oestrogen-dependent tumours. ER α was not detectable by immunohistochemistry in angiomyolipoma xenografts. In other tumours and breast cancer cell lines oestrogen-dependent Akt phosphorylation occurs in both ER α positive and negative cells (Tsai *et al.* 2001). The angiomyolipoma xenografts did express ER β and it is likely that some aspects of the oestrogen response we observed are mediated through ER β expression of ER β in angiomyolipoma has been recently reported (Boorjian *et al.* 2008).

We examined the transcriptional response to oestrogen in xenografts. Reassuringly, known oestrogen responsive genes including tumour protein D52 (Ross & Perou 2001), catenin (Chandar *et al.* 2005) and Jak 1 (Lin *et al.* 2004) were up-regulated by oestrogen in our system. In addition, ADAMTS 7, cyclin G2, jun D, TRPC1 and mannosidase α , up-regulated by oestrogen in the TD47 breast cancer cell line (Lin *et al.* 2004) were also up-regulated in our model. An interesting feature of the transcriptional response in our model was the increased expression of extracellular matrix proteins and smooth muscle related proteins not normally seen in the oestrogen response in cell lines (Frasor *et al.* 2003, Lin *et al.* 2004). The secretion of extracellular matrix proteins is likely to have a significant effect on cellular functions including growth, protection from apoptosis and activation of cell surface receptors. For example, we observed that fibronectin and collagen IV were up-regulated. These proteins protect against apoptosis in cultured ASM (Freyer *et al.* 2001) and support growth factor derived breast tumour cell proliferation *in vitro* (Haslam & Woodward 2001). Alteration of the tumour matrix to support angiomyolipoma growth is a potential mechanism contributing to oestrogen-induced tumour growth.

In angiomyolipoma xenografts the angiogenic growth factor PDGF-C was induced by oestrogen. PDGF-C is of potential relevance to angiomyolipoma and LAM as it has greater angiogenic and lymphangiogenic activity in a variety of models than other members of the PDGF family (Reigstad *et al.* 2005). As smooth muscle proliferation and angiogenesis are key features of angiomyolipoma and LAM, PDGF-C could play a role in the disease. Having reasoned that oestrogen-dependent expression of a smooth muscle mitogen may be one of the factors associated with the increased prevalence of LAM and symptomatic angiomyolipoma in women we studied PDGF-C further. We confirmed by quantitative real-time RT-PCR that *PDGF-C* was up-regulated by oestrogen in the angiomyolipoma xenografts. Importantly, active PDGF-C was also expressed by primary AML cells and

present in the smooth muscle component of human angiomyolipoma and LAM tissue. Expression was variable in tissue samples, perhaps as patients had a mixture of early and late disease and in some cases had been treated with anti-oestrogen therapies.

Interestingly, PDGF-C was not transcriptionally regulated by oestrogen in primary AML cultures. There are a number of potential reasons why this might be so. First, although both derived from angiomyolipoma, the SV7^{tert}AML cells and primary AML cells have different phenotypes and behaviour and are likely to have different transcriptional profiles. In addition, the expression of genes in a solid tumour in a whole animal treated with oestrogen for 4 weeks is likely to be different from a cell line treated *in vitro* for 1–2 days. In xenograft systems, stromal and immune cells will also respond to oestrogen leading to complex interactions between host animal and tumour leading to indirect transcriptional effects not seen in cell cultures. Consistent with this, a recent study compared the transcriptional response to oestrogen in T47D breast cancer cells in culture with T47D xenografts. Despite using the same cell line, oestrogen up-regulated 246 genes in culture and 168 in the xenograft model with only 20 genes common to both (Harvell *et al.* 2006).

The third mechanism by which oestrogen may stimulate angiomyolipoma growth is via a direct mitogenic effect. Others have shown that oestrogen can stimulate proliferation of angiomyolipoma derived cells (Yu *et al.* 2004) and angiomyolipoma related tumours (El-Hashemite *et al.* 2005). Similarly, we have shown that oestrogen can stimulate AML cell growth to a similar degree to PDGF; although it is not clear whether this is a direct or indirect effect of oestrogen. We were unable to detect a difference in BrdU incorporation, indicative of proliferation rate, of the xenograft tumours between the control and oestrogen-treated mice. This may be a consequence of incomplete penetration of BrdU into the solid tumours during the labelling period (1 h, prior to termination of the mice), resulting in superficial zones of proliferation which were not detected by this assay method, or differences in the response of the xenograft cells compared with the primary cells for the reasons outlined above.

Taken together these findings suggest that oestrogen dependent angiomyolipoma growth may occur by direct oestrogen-mediated proliferation, direct and indirect transcriptional and phosphorylation events. Anti-oestrogen treatments have been tried for LAM but not angiomyolipoma. Although no placebo controlled studies have been conducted, there is no evidence that anti-oestrogen treatments including progesterone (Johnson & Tattersfield 1999), GnRH

agonists (Harari *et al.* 2008), tamoxifen or oophorectomy (Sullivan 1998) are effective in the majority of cases. Understanding the response to oestrogen may help to design more effective treatments for angiomyolipoma and LAM.

The AML xenograft model described has some advantages over other systems for studying angiomyolipoma and LAM. Tumours can be measured non-invasively and grow reproducibly with significant changes in growth detectable within 21 days. No true model of angiomyolipoma or LAM currently exist, however, TSC-related tumours are generally studied using the Eker rat and TSC-1 or -2 knockout mice. The Eker rat has a germ line truncating mutation in TSC-2 and develops renal and uterine tumours (Yeung *et al.* 1994). TSC-1^{+/-} mice develop renal cystadenomas and carcinomas but not angiomyolipomas. These lesions develop over 3–10 months according to the system employed (Kobayashi *et al.* 2001, Wilson *et al.* 2005). TSC-2^{+/-} mice develop renal cysts and adenomas but over 15 months (Onda *et al.* 1999). Both TSC-1^{+/-} and TSC-2^{+/-} mice develop oestrogen receptor positive liver haemangiomas, which are larger and more prevalent in oestrogen-treated animals. Although the most accurate model of TSC-related hamartoma developed, these lesions also develop in most female animals over 7 months (El-Hashemite *et al.* 2005). Like these other model systems, the AML xenograft has phosphorylated S6K1 but also has the advantage of a relatively short natural history allowing experiments to be performed more quickly, possibly making it a useful tool for screening new therapies. Disadvantages of our system include the absence of significant tumour blood vessels that are seen in human angiomyolipoma. Further, the immortalised SV7^{tert}AML cell, although having constitutively active S6K1, does not have a detectable TSC mutation (Arbiser *et al.* 2001) nor do these cells express the angiomyolipoma marker HMB-45 (although recent studies have suggested a degree of variability in immunopositivity for HMB-45 in the different components of angiomyolipoma (Roma *et al.* 2007). At the present time, we and others have been unable to produce tumours with primary TSC or PEC derived xenograft systems and more physiological disease based models are required. In our study, we have addressed these shortcomings by confirming our findings in primary cells and human disease derived tissue. We consider these steps to be essential when using such model systems.

In conclusion, we have generated an angiomyolipoma xenograft model. Oestrogen treatment of animals with angiomyolipoma xenografts resulted in enhanced

tumour growth, Akt phosphorylation and a transcriptional profile characterised by the up-regulation of a wide variety gene classes. The interaction of the mTOR pathway with oestrogen signalling is likely to be important in diseases with a female predominance including angiomyolipoma, LAM, uterine myomata and breast cancer. Further work and better models are required to understand the role of oestrogen in the mTOR pathway in angiomyolipoma, LAM and TSC.

Declaration of interest

No conflict of interest that could be perceived as prejudicing the impartiality of the research reported in this article has been reported by any of the authors.

Funding

The study was funded by LAM Action (grant reference RB23BP), the LAM Foundation (grant number LAM050-FP01-05) and Nottingham University Hospital Special Trustees (grant number STR 45/02/M).

Acknowledgements

We are grateful to Mr John Ronan, Drs Phillip Clarke, Lorna Magowan and Charlotte Billington for technical assistance and Urological surgeons Mr Bishop, Mr Doble and Mr Wallace for providing the operative specimens.

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