A VEGF/JAK2/STAT5 axis may partially mediate endothelial cell tolerance to hypoxia

Andrew C. DUDLEY¹, David THOMAS, James BEST and Alicia JENKINS

Department of Medicine, St. Vincent's Hospital, University of Melbourne, Corner of Princes and Regent Streets, Fitzroy, VIC, 3065, Australia

Perturbation of oxygen flow occurs in disease states such as diabetic retinopathy and cancer. To maintain oxygen homeostasis, the mammalian microvascular endothelium undergoes a dramatic reorganization to assist in bringing oxygen and nutrients to oxygen-starved tissues. This process is termed angiogenesis and is common in certain cancers with hypoxic foci and in areas of focal ischaemia in the diabetic retina. In the present study, we report on the activation of the JAK2/STAT5 pathway (where JAK stands for Janus kinase and STAT stands for signal transduction and activator of transcription) by low oxygen in microvascular endothelial cells. This activation appears to occur downstream of

VEGF (vascular endothelial growth factor), a well-known pro-angiogenic factor, and is related to repression of proapoptotic FAS(CD95)/FASL(CD95L). These results indicate that the JAK/STAT pathway may play a pivotal role during tumour-associated or retinal angiogenesis in which endothelial cell survival during tissue hypoxia is critical for maintaining either the growth of neoplasms or the inappropriate retinal neovascularization common in diabetic retinopathy.

Key words: angiogenesis, endothelial cell, FAS/FASL, hypoxia, JAK/STAT, vascular endothelial growth factor (VEGF).

INTRODUCTION

Many tumours have a high metabolic rate combined with a chaotic, variable blood flow which limits diffusion of oxygen and other nutrients from an adjacent vascular bed – for these reasons, they become hypoxic [1]. Thus, for most tumours to continue growing, they must recruit new blood vessels. This is typically achieved through the action of hypoxia-inducible paracrine mediators such as VEGF (vascular endothelial growth factor), which, in a process termed angiogenesis, facilitates the reorganization of the pre-existing vasculature into a network of *de novo* microvessels [2]. These new microvessels can then freely infiltrate the growing tumour supplying it with nutrients and oxygen. Similarly, in diabetic retinopathy, tissue hypoxia stimulates the release of VEGF and other soluble factors, resulting in uncontrolled retinal neovascularization [3]. Angiogenesis in both retinopathy and cancer are remarkably similar as the new vessels in each instance are characterized by vessel haemorrhages, poorly formed and tortuous endothelial cell tubes, absence of pericytes, and endothelial cell hyperpermeability [4].

Since the identification of angiogenesis as a process underlying disease states such as cancer and diabetes, numerous studies have been instigated to elucidate the underlying molecular pathways that regulate how endothelial cells respond to an angiogenic stimulus like hypoxia. One notable feature of endothelial cells is their ability to tolerate prolonged periods without oxygen [5–7]. For example, endothelial cells within the interior of vascularized, solid tumours may encounter pO₂ levels as low as 0.5 mmHg (1 mmHg = 0.133 kPa), yet they still somehow survive [1]. This indicates that endothelial cells have evolved to survive in the face of hypoxia such as might occur during normal tissue injury, and that during disease states like cancer and diabetes, endothelial cell survival could potentiate tumour growth or retinal neovascularization respectively. Many studies have now recog-

nized the importance of VEGF in endothelial cell growth and survival during angiogenesis, and strategies to inhibit selectively VEGF signalling offer promise for the treatment of certain cancers and diabetic retinopathy [8].

Recent studies have determined that the JAK (Janus kinase)/STAT (signal transduction and activators of transcription) pathway, a cell-signalling cascade often associated with immune function, is involved in mediating processes related to angiogenesis. For example, VEGF-activated STAT3 was required for endothelial cell tube formation in a human *in vitro* model [9] and IL-3 (interleukin 3)-stimulated endothelial cell migration involved STAT5 [10]. The JAK/STAT pathway comprises a family of four non-receptor tyrosine kinases (JAKs) and seven 85–95 kDa transcription factors (STATs) that are regulated by phosphorylation on specific serine and tyrosine residues [11]. Typically, the JAK/STAT pathway is activated by type II IFNs (interferons), interleukins or other cytokines whose receptors lack intrinsic kinase activity. Surprisingly, VEGF, whose receptor is a receptor tyrosine kinase, has also been shown to activate specific STATs. For example, VEGF was shown to cause phosphorylation of STAT1 and STAT6 [12] and, recently, VEGF was shown to activate STAT3 and STAT5 in models of ovarian carcinoma [13,14]. These results support a role for STAT 3 and STAT 5 in cancer where they have previously been shown to be constitutively active [15,16].

In addition to their potential role in angiogenesis and responses to VEGF, STATs are sensitive to other cellular stressors [17]. Some of the more well-characterized stressors include UV light [18], hyperosmolarity [19], reactive oxygen species [20] and high glucose [21], thus implicating STATs in processes related to diabetes. In cardiomyocytes undergoing hypoxia–reperfusion injury, STAT1 propagates a proapoptotic signal leading to cardiomyocyte death [22–25], whereas STAT5 seems to fulfil the opposite role and activates survival signals [26]. For these reasons,

Abbreviations used: BRCEC, bovine retinal capillary endothelial cells; FBS, fetal bovine serum; GAS, gamma-activated site; HCM, hypoxia-conditioned medium; HSP70, heat-shock protein 70; IFN, interferon; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; NP40, Nonidet P40; RT, reverse transcriptase; STAT, signal transduction and activator of transcription; VEGF, vascular endothelial growth factor.

¹ Present address and address for correspondence: Harvard Medical School, Karp Family Research Laboratories, 12th Floor, Children's Hospital, 300 Longwood Ave., Boston, MA 02115-5737, U.S.A. (email andrew.dudley@childrens.harvard.edu).

STATs are thought of as having 'yin and yang'-type properties whereby, depending on the type of cellular stressor, either cell-death or cell-survival pathways are activated [27].

Despite the growing number of reports investigating a role for the JAK/STAT pathway in cardiomyocytes undergoing hypoxia-reperfusion, to our knowledge no studies have examined a role for the JAK/STAT pathway in endothelial cells exposed to low oxygen, which is an important stimulus for angiogenesis. Therefore, in the present study, we investigated JAK/STAT phosphorylation and activation of STAT-dependent target genes in microvascular endothelial cells exposed to hypoxia or anoxia. It was determined that the JAK/STAT pathway is activated by lowered oxygen, and that this activation is related to inhibition of a cellular proapoptotic pathway. These results indicate that the JAK/STAT pathway may play an important role in endothelial cell survival during tissue hypoxia and angiogenesis, which are characteristics of both diabetes and cancer.

MATERIALS AND METHODS

Cell culture and media

BRCECs (bovine retinal capillary endothelial cells) were isolated and characterized as described previously [28]. The cells were maintained in a 37 °C incubator with 5 % CO₂ and used between passages 3 and 12. MCDB131 (Life Technologies, Gaithersburg, MD, U.S.A.) containing 10 % (v/v) FBS (fetal bovine serum), 2 mM L-glutamine and 1000 units/l penicillin/streptomycin/ampicillin was the growth medium. Hypoxia was achieved by placing the cells in a modular incubator chamber (Billups-Rothenberg, Del Mar, CA, U.S.A.) that was then flushed for 5 min (20 litres/min) with a gas mixture of 90 % N₂, 5 % CO₂ and 5 % O₂. Anoxia was achieved by flushing the chamber with a gas mixture of 95 % N₂ and 5 % CO₂. These conditions are reported by the manufacturer to render the hypoxia chamber completely purged.

Determination of cell numbers

Cell numbers were determined by dispersing the cells in trypsin and counting by a Coulter counter (Model Z1; Beckman Coulter, Palo Alto, CA, U.S.A.). These experiments were performed in triplicate in 24-well plates.

FACS analysis

After treatment with anoxia, cells were dispersed in trypsin, washed with PBS containing 0.1 % FBS and then fixed in 80 % (v/v) ethanol for 20 min on ice. Cells were then washed twice with PBS/0.1 % FBS and resuspended in a PBS/0.2 % Triton X-100 solution containing 1 µg/ml RNase and 50 µg/ml propidium iodide. Samples were incubated in the dark at room temperature (25 °C) for 20 min before FACS analysis.

Preparation of whole cell lysates and cytosolic and nuclear fractions

Cells were washed twice with PBS containing 2 mM sodium orthovanadate and whole cell lysates were prepared in RIPA extraction buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1 % NP40 (Nonidet P40) 0.5 % sodium deoxycholate and 1 % SDS] on ice for 15 min. After centrifugation at 14 000 g for 15 min (4 °C), a small amount was removed and the protein concentration was determined by the modified Lowry assay (Bio-Rad, Hercules, CA, U.S.A.). Crude cytosolic and nuclear fractions were prepared by resuspending cell pellets in 100 µl of sucrose buffer (0.32 M sucrose, 10 mM Tris, pH 8.0, 3 mM CaCl₂, 2 mM MgOAc,

0.1 mM EDTA and 0.5 % NP40), which were then centrifuged at 500 g for 5 min (4 °C). The supernatant (cytosolic fraction) was saved and the pelleted nuclei were washed in sucrose buffer without NP40 and then centrifuged as before. Cells were then resuspended in 15 µl of low-salt buffer (20 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 25 %, v/v, glycerol and 0.5 mM PMSF) and an equal volume of high-salt buffer (20 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 800 mM KCl, 0.2 mM EDTA, 25 % glycerol and 1 % NP40) was slowly added. The mixture was incubated on ice for 30 min with occasional mild vortex-mixing. At the end of the 30 min incubation, samples were centrifuged at 14 000 g for 15 min (4 °C). The supernatant (nuclear extract) was transferred to a fresh tube and the protein concentration was determined by the modified Lowry assay. An equal volume of 2× sample buffer was added, and the samples were heated to 95 °C for 5 min before electrophoresis and Western blotting.

SDS/PAGE and Western blotting

Equal amounts of protein (20–30 µg) were subjected to SDS/PAGE (8 % polyacrylamide) and then transferred on to PVDF membranes. After blocking the membrane for 1 h [in TBST (50 mM Tris, 0.5 % Tween 20 and 300 mM NaCl, pH 7.5) containing 5 % BSA for all phospho-specific antibodies or TBST containing 5 % (w/v) skimmed milk powder for all other antibodies], the primary antibodies were added and left overnight at 4 °C. On the next day, the membrane was washed three times with TBST, and the secondary antibodies were added for an additional hour. After three more washes with TBST, membranes were incubated with enhanced chemiluminescence substrate and then exposed to a radiographic film according to the manufacturer's instructions (Pierce, Rockford, IL, U.S.A.).

Reverse transcription PCR

Total cellular RNA was extracted with TRIzol® (Invitrogen) and the concentration was determined by spectrophotometry. RNA (3–4 µg) was subjected to RT (reverse transcriptase)-PCR by a standard method. cDNA was then amplified by PCR (25–30 cycles) using the primers listed in Table 1 and a touchdown PCR procedure.

Pull-down assay with FAS(CD95)/GAS oligonucleotide

Double-stranded oligonucleotides based on the GAS (gamma activated site) sequence from the FAS promoter were 3'-end-labelled with biotin (5'-TTCATATGGTTAACTGTCCATTCCAGGAACGTCTGTGAGC[3BTN]-3'). Oligonucleotide (5 µg) was mixed with 500 µg of whole cell lysate [prepared in single detergent lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl and 1 % NP40)] for 20 min on ice. Streptavidin beads (15 µl) were then added and the samples were incubated for an additional 15 min on ice. The samples were centrifuged for 1 min (5000 g at 4 °C) and then washed four times with single detergent lysis buffer. Finally, 2× sample buffer was added and the samples were heated to 95 °C for 5 min before electrophoresis and Western blotting.

Immunofluorescence

Cells were washed twice with PBS and then fixed for 10 min at room temperature in 4 % (w/v) paraformaldehyde. After two additional washes with PBS, the cells were permeabilized for 5 min with PBS containing 0.1 % Triton X-100. The cells were then incubated with primary antibodies overnight at 4 °C (in PBS containing 0.1 % BSA). On the next day, cells were washed three times with PBS and then stained with FITC-conjugated secondary antibodies for an additional 2–3 h at room temperature.

Table 1 Oligonucleotides for PCR amplification

Primer name	Sequence (5'–3')	Expected size
VEGF	CGAACCACATGAACCTTCTGC CCTCAGTGGGCACACACTCC	302
Bcl-xL	CGGATAGCCCTGCTGTGAA TGCTGCATTGTTCCCGTAGA	450
Bcl-2	GGGAACAGGCTACGATAACCG AAAGAAGGCCACGATGCG	432
Bax	AGGATCGAGCAGGGCGAAT CACTGTCTGCCATGTGGGTG	519
Caspase 4	GATGTTGGAATCTTGGGCAA CCTGGGAGGCATATGATCAA	465
HSP70	ATGGCTATCGGCATCGACC CGCTGCGAGTCGTGAAGT	473
HSP90	AGGAGGAGGTGGAGACTTTC GACGGTCACTTCTCAGCCAC	459
IFN γ	TGGTTCTTATGGCCAGGGC CGGCCTCGAAAGAGATTCTG	494
IFN α	GGGTCTGATGCTCTGAGA ACAACCTCCAGGCACAAGG	431
36B4	CGACCTGGAAGTCCAACATC ATCTGCTGCATCTGCTTG	109
IRF	TCACTCGGATGCGCATGA CTTGGCCTTGCTCCTAGCGT	408
FAS	TGTCCGGGATCTGGGTTC CGTGGTGCAAGGGTTACAGT	435
FASL	GAGAGTCCACCAGCCAAAGG TGCTTCCCATTCCAAAGG	151

After 2–3 more washes with PBS, cells were mounted with Fluoromount G and then visualized by fluorescence microscopy. Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole) for 5 min before viewing.

Buffers and antibodies

The following buffers were used for experimental analyses: TBST, sucrose buffer, low-salt buffer, high-salt buffer, single detergent lysis buffer and RIPA buffer. The phospho-specific and pan anti-STAT, anti-JAK and anti-MAPK (mitogen-activated protein kinase) antibodies were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.) with the exception of the pan anti-STAT5 antibody, which was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and the pan anti-JAK2 and anti-pSTAT1 (S727) antibodies, which were purchased from Upstate Biotechnology (Charlottesville, VA, U.S.A.). The blocking antibody to VEGFA was purchased from Santa Cruz Biotechnology.

RESULTS

BRCEC are comparatively resistant to anoxia

Using VEGF as an indicator, it was found that complete anoxia, but not hypoxia (5% O₂), resulted in the increased expression of VEGF at the protein (Figure 1A) and mRNA (Figure 1B) levels after 24 h. In cell growth studies, while 3 days of continuous anoxia resulted in striking changes in BRCEC phenotype (Figure 1C) and a 20% reduction in growth (Figure 1D), 5 days of continuous hypoxia produced no such effect (Figure 1E). The changes in cell number were related to a reduction in the number of cells in S-phase, G₂ arrest and an increased number of cells in the

sub-G₁ fraction (Figure 1F). In contrast with BRCEC, other cell types were markedly more sensitive to 3 days of complete anoxia, demonstrating a 60–70% reduction in growth, cell rounding and detachment (Figure 1G). Taken together, these results indicated that anoxia does cause moderate inhibition of BRCEC growth and must activate pathways leading to senescence, but these cells are remarkably tolerant to a low oxygen environment.

Anoxia results in the phosphorylation of JAK2, STAT1 (S727) and STAT5 (Y694)

BRCEC were cultured under normoxia, hypoxia and anoxia for the indicated times and JAK/STAT phosphorylation was determined in whole cell lysates by Western blotting. The results showed that hypoxia caused a moderate increase in JAK2 phosphorylation after 24 and 48 h, whereas anoxia caused a robust increase in JAK2 phosphorylation as early as 6 h, which continued through the 48 h time course. Slight increases in the total levels of JAK2 were also observed during anoxia (Figure 2A). Densitometry was performed with the JAK2 blot and the ratios of pJAK2/total JAK2 are depicted above Figure 2(A). No increase in TYK2 phosphorylation during hypoxia or anoxia was detected (results not shown). STAT1 (S727) phosphorylation was found to increase at all time points of anoxia, with only a slight increase during hypoxia. Despite the overall levels of STAT3 being somewhat variable, no time-dependent increase in STAT3 (Y705) phosphorylation was evident. Like JAK2, STAT5 (Y694) phosphorylation was strikingly increased by anoxia as early as 6 h, which continued throughout the 48 h time course (Figure 2B). STAT5 phosphorylation was transient and peaked at 24 h but had still not returned to basal levels by 48 h. A similar increase in STAT5 phosphorylation was observed in HUVEC (human umbilical-vein endothelial cells) during anoxia (Figure 2C). No STAT1 (Y701) phosphorylation was detected under any of the test conditions (results not shown).

Anoxia results in the nuclear translocation of JAK2 and STAT5

Next, BRCEC were exposed to anoxia for different times (6, 24 or 48 h) and cell lysates were fractionated into crude cytosolic, nuclear or insoluble nuclear fractions. JAK/STAT phosphorylation was measured by Western blotting. The results showed that, consistent with previous findings, JAK2 phosphorylation was increased in the cytosolic fraction during anoxia. Surprisingly, JAK2 was detected in the nuclear fraction during anoxia (Figure 3A). Whereas STAT1 (S727) phosphorylation was increased by anoxia in the cytosolic compartment as before, there was no evidence of nuclear accumulation (Figure 3B). Likewise, there was no increase in STAT3 (Y705) phosphorylation or nuclear accumulation as shown previously. STAT5 (Y694) phosphorylation was increased in the cytosolic fractions and, like JAK2, STAT5 was found to accumulate in the nucleus after anoxia with a time course very similar to JAK2. Furthermore, JAK2 and STAT5 were found to associate physically during anoxia (Figure 3C). A small aliquot of cell lysate (20% input) was also subjected to Western-blot analysis to demonstrate that the total levels of STAT5 remained unchanged in Figure 3(C). Immunofluorescence studies confirmed the nuclear accumulation of STAT5 (Y694) during anoxia (Figure 3D). Taken together, these results indicated that anoxia causes JAK2 and STAT5 phosphorylation and nuclear translocation.

BRCEC response to anoxia and STAT-dependent gene activation

In cardiomyocytes undergoing hypoxia–reperfusion, STAT1 is phosphorylated on S727 by p38, resulting in the downstream

Figure 1 BRCEC are comparatively resistant to anoxia

(A) VEGF Western-blot analysis and RT-PCR (B) in hypoxia- and anoxia-treated BRCEC. 36B4 was used as the house-keeping gene for PCR. (C) Anoxia (24 h) strikingly alters BRCEC phenotype, but very few cells round up and detach. Anoxia for 48 h decreases BRCEC growth by approx. 20% (D), but up to 5 days of continuous hypoxia (E) does not affect BRCEC growth (* $P < 0.05$, results are statistically different from control). Cell-cycle analysis was performed by propidium iodide staining and FACS (F). Compared with other cell types, BRCEC are less sensitive to the growth inhibitory effects of anoxia (48 h) (G).

activation of FAS [24]. It was questioned whether this might also occur in BRCEC because an increase in STAT1 (S727) phosphorylation during anoxia was detected. Under the test conditions, no increase in p38 phosphorylation was detected during anoxia or hypoxia (Figure 4A). However, a transient increase in p44/p42 phosphorylation and a sustained increase in SAPK (stress-activated protein kinase)/JNK (c-Jun N-terminal kinase) phosphorylation was detected in anoxia-treated cells. No change in STAT1 (S727) phosphorylation was detected when MAPK inhibitors or a JAK2 inhibitor (AG490) were added before anoxia (Figure 4B).

Next, it was asked whether STAT1-dependent target genes might be altered by anoxia. The results showed that there was no change in antiapoptotic genes such as Bcl-2 and Bcl-xL [29] (Figure 4C) and no change in other genes including HSP70 (heat-shock protein 70) or HSP90 [30] in BRCEC undergoing anoxia treatment (Figure 4D). Furthermore, no change in a selection of

STAT-dependent proapoptotic genes was detected with the exception of FAS and FASL (CD95L) that showed a moderate trend towards decreasing expression during anoxia (Figure 4E). To confirm these results, the anoxia time course was extended to 72 h. Consistent with previous findings, FAS and FASL mRNAs were both found to decrease time-dependently during anoxia (Figure 4F). Taken together, these results suggested that endothelial cells respond differently to low oxygen compared with other cell types like cardiomyocytes where FAS is dramatically increased by hypoxia-reperfusion [31].

FAS is decreased by anoxia but its transcription is de-repressed by the JAK2 inhibitor AG490

We next examined what effect JAK2 inhibition might have on FAS expression during anoxia or normoxia. As shown in Figure 5(A), 10 μ M AG490 time-dependently down-regulated JAK2

Figure 2 Anoxia results in the phosphorylation of JAK2, STAT1 (S727) and STAT5 (Y694)

Western-blot analysis in whole cell lysates from hypoxia- or anoxia-treated BRCEC. (A) JAK2 phosphorylation and (B) STAT1, STAT3 and STAT5 phosphorylation. (C) STAT5 phosphorylation in HUVEC cell lysates after anoxia. After probing with each phospho-specific antibody, blots were stripped and reprobed with pan anti-STAT1, anti-STAT3 or anti-STAT5 antibodies.

phosphorylation during normoxia, demonstrating that AG490 inhibits JAK2 phosphorylation and thus its activity. In addition, AG490 inhibited the phosphorylation of STAT5 during anoxia in both the cytosol and nucleus (Figure 5B). Finally, when BRCEC were pretreated with AG490 before 72 h of anoxia, both FAS and FASL were completely derepressed (Figure 5C). This was despite the fact that AG490 had no inhibitory effect on FAS/FASL or other genes during normoxia. Taken together, these results suggested that JAK2, when activated during anoxia, must play a role in repressing FAS/FASL transcription, potentially through downstream activation of STAT5.

STAT5 binds to the GAS in the FAS promoter

The FAS promoter contains a GAS, and STAT3 has previously been shown to repress constitutive FAS transcription [32]. However, a role for STAT5 in this capacity has not been described. Initially, we performed gel-shift experiments using the GAS core-binding sequence from the FAS promoter or an oligonucleotide containing three tandem repeats of the GAS along with anoxia-

treated BRCEC. These experiments were unsuccessful as no definitive STAT binding could be detected. However, using a biotinylated oligonucleotide derived from the FAS promoter, STAT5 binding could be detected. Figure 6(A) shows a pull-down experiment using the biotinylated oligonucleotide and normoxia- or hypoxia-treated BRCEC lysates. While no STAT1 binding could be detected by Western-blot analysis, STAT5 was bound constitutively, but anoxia treatment had no effect on STAT5 binding. Finally, pretreatment with AG490 had only a marginal effect on STAT5 binding to the FAS promoter *in vitro* (Figure 6B). A small aliquot (20% input) was subjected to Western-blot analysis to demonstrate that the total levels of STAT5 remained unchanged. These results suggest that STAT5 does bind to the FAS promoter *in vitro*, but this binding does not appear to be entirely dependent on STAT5 phosphorylation.

HCM (hypoxia-conditioned medium) recapitulates the effects on JAK2/STAT5 phosphorylation, which is reversed in the presence of a VEGF-blocking antibody

Finally, it was asked if the effects of anoxia on JAK/STAT phosphorylation occurred downstream of a soluble, secreted factor. To answer this question, media from anoxia-treated BRCEC (24 h) were plated on to BRCEC left in normoxia. The cells were then incubated for an additional 24 h. In addition, a VEGF-blocking antibody or an isotype control antibody was added where noted. The results showed that HCM alone was sufficient to recapitulate the effects of anoxia on both JAK2 and STAT5 phosphorylation (Figure 7A). Furthermore, this effect could be reversed when VEGF was blocked with a polyclonal antibody specific for VEGFA. HCM also caused a down-regulation of FAS expression similar to what was observed with anoxia alone, and this effect could be reversed with the VEGF-blocking antibody. In separate experiments, the direct addition of rVEGF165 resulted in a time-dependent increase in both JAK2 and STAT5 phosphorylation (Figure 7B) and resulted in the down-regulation of FAS mRNA when measured with RT-PCR (Figure 7C). Accordingly, when BRCEC were supplemented with exogenous VEGF, their survival was augmented during anoxia (Figure 7D). These results indicated that the effects of anoxia on FAS transcription and on JAK2/STAT5 phosphorylation at least partially occur downstream of VEGF.

DISCUSSION

Tissue hypoxia is characteristic of solid tumours and blinding ocular disorders such as diabetic retinopathy. For example, retinal arterial occlusion renders the entire inner retina anoxic [33], and pO₂ levels in implanted human tumour xenografts in SCID mice averaged 8.3 mmHg to values below 0.5 mmHg (normal tissue values average, 20–40 mmHg) [34]. Importantly, pO₂ levels were decreased not only in the tumour mass, but also in the tumour-associated blood vessels, indicating that the endothelial cells themselves are exposed directly to hypoxia *in vivo*. As Scheurer et al. [35] have pointed out, these results imply that the endothelial cells are critically involved in oxygen sensing and most probably contribute to the onset of the angiogenic response. Although it is well known that VEGF is a survival factor for endothelial cells in culture, it is speculated that the VEGF-secreting tumours may potentiate the survival of tumour-associated endothelial cells in neoplasms. It is probable that the hypoxic microenvironment within some tumours may also lead to an increase in VEGF expression in the endothelial cells themselves, resulting in an autocrine mechanism to prolong endothelial cell survival.

Figure 3 Anoxia results in the nuclear translocation of JAK2 and STAT5

Cytosolic and nuclear fractions from anoxia-treated BRCEC were subjected to Western-blot analysis. **(A)** JAK2 phosphorylation and nuclear translocation and **(B)** STAT1, STAT3 and STAT5 phosphorylation and nuclear translocation. β -Actin was used as a control to show the absence of cross-contamination between cytosol and soluble nuclear fractions. **(C)** JAK2/STAT5 co-immunoprecipitation from normoxia- or anoxia-treated lysates. A small aliquot of each sample was run alongside the immunoprecipitation and blotted with pan anti-STAT5 antibodies. **(D)** Immunofluorescence confirms the nuclear localization of STAT5 (Y694) during anoxia. pSTAT (Y694) was detected with an FITC-conjugated goat anti-rabbit secondary antibody. The nuclei were stained with DAPI.

To our knowledge, this is the first study to investigate the phosphorylation status of JAKs or STATs in microvascular endothelial cells exposed to low oxygen, but there have been several reports of this type in cardiomyocytes undergoing hypoxia-reperfusion. For example, a report by Stephanou et al. [24] indicates that, in cardiomyocytes, STAT1 is phosphorylated on S727, leading to FAS activation and apoptosis during hypoxia-reperfusion. Our results in endothelial cells suggest that STAT1 does undergo phosphorylation during anoxia (S727), but this does not appear to result in the activation of proapoptotic genes like FAS or FASL. In fact, we observe the opposite trend that both FAS and FASL mRNAs are time-dependently decreased by low oxygen. This difference may arise due to the different physiological consequences of hypoxia versus hypoxia-reperfusion or due to the fact that endothelial cells and cardiomyocytes are fundamentally different cell types.

Yamaura et al. [26] have hypothesized the existence of a survival signal propagated by JAK2/STAT5 in cardiomyocytes undergoing hypoxia-reperfusion. It is believed that we have potentially uncovered this prosurvival signal, which is the repression of FAS/FASL. FAS expression appears to be linked to the JAK/STAT

pathway specifically through JAK2 and STAT5 because AG490 de-represses FAS during anoxia, it inhibits JAK2 and STAT5 phosphorylation and STAT5 is physically associated with the FAS promoter *in vitro*. Studies demonstrating that inhibition of constitutive JAK2 results in apoptosis and down-regulation of STAT5 transcriptional activity are consistent with our results showing that proapoptotic FAS is up-regulated when activated JAK2 (during anoxia) is inhibited [36]. Supporting a role for FAS/FASL in processes related to angiogenesis, recent studies have shown that dermal and retinal microvascular density was increased in mice deficient for FAS/FASL [37] and an antibody against FAS prevented vascular tube formation in matrigel plugs [38].

It is not yet clear how STAT5 might impart its effects on FAS expression. STAT5 was bound constitutively to the FAS/GAS oligonucleotide and AG490 did not dramatically affect STAT5 binding. These results suggest that STAT5's association with the FAS promoter is not entirely dependent on STAT5's phosphorylation. However, STAT5's inhibitory effect on FAS transcription may be dependent on STAT5 phosphorylation possibly by facilitating STAT5's physical coupling with the promoter sequence (thus rendering FAS repressed) or by allowing STAT5 to interact

Figure 4 Anoxia, MAPK and STAT-dependent gene activation in BRCEC

(A) p38, p44/p42 and SAPK/JNK phosphorylation in hypoxia- or anoxia-treated whole cell lysates. (B) The p38 inhibitor (SB203580), MEK inhibitor (PD98059) and the JAK2 inhibitor (AG490) do not block STAT1 (S727) phosphorylation during anoxia. SB203580 was used at 5 μ M, AG490 at 10 μ M and PD98059 at 50 μ M. All inhibitors were added 30 min before anoxia (24 h). RT-PCR analysis of STAT-dependent antiapoptotic genes (C), HSP70 and HSP90 (D) and proapoptotic genes (E). (F) Extended time course for FAS and FASL in cells undergoing anoxia treatment shows the down-regulation of FAS and FASL.

with a co-repressor protein. We were unable to demonstrate a change in affinity of STAT5 for the FAS promoter sequence *in vitro* during anoxia, but this may be due to the fact that whole cell lysates were used, where the total amounts of STAT5 are unchanged during normoxia compared with anoxia. It is expected that using nuclear extracts, where a small amount of STAT5 is detectable in the nucleus during anoxia but not normoxia (see Figure 5B), would reveal that the levels of STAT5 bound to the FAS promoter are increased by low oxygen. Preliminary experiments done in this way have proven to be difficult, and we suspect that due to the low levels of STAT5 in the nucleus during anoxia, there may be problems with sensitivity using the pull-down assay and Western blotting. For reasons not yet clear, preliminary gel-shift analysis (which is a more sensitive assay compared with Western blotting) in our laboratory did not demonstrate a distinct STAT binding to the FAS/GAS sequence that was insensitive to competition by 100-fold excess of unlabelled probe. Thus, to make any solid conjecture with regard to which STAT(s) might be binding to the FAS/GAS sequence, gel-shift analysis needs to be performed and

optimized with appropriate controls and our results so far using biotinylated oligonucleotides must be considered as preliminary.

It was interesting to note that we could detect nuclear translocation of JAK2 in anoxia-treated cells. Constitutive JAK nuclear localization has recently been demonstrated to be negligible [39], whereas other reports have demonstrated significant JAK2 in the nucleus [40]. Some of these reports are questionable due to possible non-specific cross-reactivity of the anti-JAK antibodies as has been clearly demonstrated by Behrmann et al. [39]. Overall, our results support an absence of constitutive JAK2 in the soluble nuclear fraction, but clear nuclear staining for JAK2 was evident in anoxia-treated cells by Western-blot analysis. JAK2 has no known nuclear import signal, so how JAK2 enters the nucleus remains to be answered. It is possible that JAK2 is imported via its association with STAT5 because we demonstrated an enhanced protein-protein interaction between JAK2 and STAT5 in anoxia-treated cells and the time dependence of JAK2 and STAT5 nuclear accumulation was nearly identical in Western blotting experiments.

Figure 5 FAS is decreased by anoxia but its transcription is de-repressed by the JAK2 inhibitor AG490

(A) Pretreatment with 10 μ M AG490 for 30 min time-dependently decreases JAK2 phosphorylation in whole cell lysates during normoxia. (B) STAT5 phosphorylation is decreased in the cytosolic and nuclear fractions during anoxia following pretreatment with 10 μ M AG490. (C) Both FAS and FASL are derepressed when 10 μ M AG490 is added 30 min before subjecting cells to anoxia (72 h).

VEGF was only recently demonstrated to play a role in STAT activation in a pathological milieu such as cancer [13,14]. Our results extend these findings and support a link between STAT5 and VEGF and bridge the gap between VEGF-mediated repression of a proapoptotic pathway during hypoxia. For example, although FAS itself was not measured, other studies demonstrating an inverse correlation between FASL and VEGF [41] and down-regulation of FASL by addition of exogenous VEGF [42] support the notion that VEGF is playing a dominant role as a survival factor in the endothelium during angiogenesis. Furthermore, it was recently reported that blocking the erythropoietin receptor resulted in down-regulation of phosphorylated JAK2 and STAT5 and increased apoptosis in tumour endothelial cells [43]. This report establishes an intriguing link between the JAK/STAT pathway and survival of the tumour endothelium during angiogenesis. If our interpretation is correct, we predict that blocking STAT5 specifically in tumour-related endothelial cells or in retinal capillary endothelial cells in the hypoxic microenvironment should enhance endothelial cell apoptosis because FAS would be de-repressed. Thus specific inhibition of STAT5 could be another downstream target for blocking unwanted tumour-associated or retinal angiogenesis.

Figure 6 STAT5 binds to the GAS in the FAS promoter

(A) Pull-down assay using biotinylated oligonucleotide containing the GAS sequence from the FAS promoter. After the binding reaction, samples were washed and subjected to electrophoresis followed by Western blotting with a pan anti-STAT1 or anti-STAT5 antibody. (B) AG490 (25 μ M) only marginally reduces STAT5 binding to the FAS promoter during 24 h of anoxia in whole cell lysates.

It has become clear that STAT3 and STAT5 play a role in the pathogenesis of cancer and possibly other diseases. Numerous reports have demonstrated that constitutively active STAT3 is present in tumour cells, is required for and enhances transformation, and inhibits apoptosis, thus fulfilling STAT3's role as an oncogene [44,45]. Other studies have also demonstrated a prosurvival role for STAT5 in various types of tumour cells [46]. The role of STATs in cancer has recently been comprehensively reviewed and, as the authors point out, it remains to be determined if STAT5 can contribute to malignant properties other than cell proliferation and survival, such as angiogenesis [47]. Here, we believe that a possible role for STAT5 in processes related to angiogenesis has been established, thus strengthening the hypothesis that STAT5 might contribute indirectly to diseases such as cancer and diabetic retinopathy.

Hypoxia plays such a large role in the pathophysiology of disease that entire conferences are now devoted to this topic. In the present study, we have reported on the effects of lowered oxygen on the growth of microvascular endothelial cells. Our findings suggest a potential role for a VEGF/JAK2/STAT5 axis impinging on the transcription of FAS, a well-characterized proapoptotic gene. We believe these results to be relevant to models of angiogenesis in diabetic retinopathy and cancer where endothelial cell survival is critical during neovascularization. The challenge

Figure 7 HCM recapitulates the effects of anoxia alone on JAK2 and STAT5 phosphorylation

(A) Conditioned medium from BRCEC grown in anoxia (24 h) was added to BRCEC grown in normoxia and left for an additional 24 h. As a control, the medium alone was placed under anoxia (cell-free). Cell lysates were prepared for Western blotting (WB) and RT-PCR. A VEGF-blocking antibody (2.5 μ g/ml) or an isotype control antibody was also included in the experiment. (B, C) Addition of VEGF alone (50 ng/ml) resulted in JAK2 and STAT5 phosphorylation and the down-regulation of FAS mRNA (48 h). (D) Adding 50 ng/ml exogenous VEGF augmented BRCEC survival during anoxia compared with normoxic controls (48 h).

now is to develop specific inhibitors of STATs that could one day translate into the clinical setting.

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