Cancer Microenvironment (2008) 1:131–139
DOI 10.1007/s12307-008-0013-4

ORIGINAL PAPER

Microenvironment Changes (in pH) Affect VEGF Alternative Splicing

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Received: 10 April 2008 / Accepted: 4 July 2008 / Published online: 29 July 2008

Abstract Vascular endothelial growth factor-A (VEGF-A) has several isoforms, which differ in their capacity to bind extracellular matrix proteins and also in their affinity for VEGF receptors. Although the relative contribution of the VEGF isoforms has been studied in tumor angiogenesis, little is known about the mechanisms that regulate the alternative splicing process. Here, we tested microenvironment cues that might regulate VEGF alternative splicing. To test this, we used endometrial cancer cells that produce all VEGF isoforms as a model, and exposed them to varying pH levels, hormones, glucose and $CoCl_2$ (to mimic hypoxia). Low pH had the most consistent effects in inducing variations in VEGF splicing pattern (VEGF121 increased significantly, $p<0.001$, when compared to VEGF145, 165 or 189). This was accompanied by activation of the p38 stress pathway and SR proteins (splicing factors) expression and phosphorylation. SF2/ASF, SRp20 and SRp40 down-regulation by siRNA impaired the effects of pH stimulation, blocking the shift in VEGF isoforms production. Taken together, we show for the first time that acidosis (low pH) regulates VEGF-A alternative splicing, may be through p38 activation and suggest the possible SR proteins involved in this process.

Keywords Alternative splicing · Endometrial carcinoma · Microenvironment · SR proteins · VEGF

Abbreviations

ARE	AU-rich elements
DMEM	Dulbecco's modified Eagle's medium
ECL	enhanced chemiluminescence
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
hnRNP	heterogeneous nuclear ribonucleoprotein
HuR	hypoxia-induced stability factor
MAPK	mitogen-activated protein kinase
PAIP2	poly(A)-binding protein-interacting protein 2
RQ-PCR	real time RT-PCR
RRM	RNA recognition motif
RS domain	domain rich in alternating serine and arginine residues
SAPK/JNK	stress-activated protein kinase/ Jun-amino-terminal kinase
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SRp	serine/arginine-rich protein
UTR	untranslated region
VEGF	vascular endothelial growth factor

Electronic supplementary material The online version of this article (doi:10.1007/s12307-008-0013-4) contains supplementary material, which is available to authorized users.

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Introduction

Angiogenesis is important for the expansion of solid and hematologic cancers. In response to metabolic changes occurring within the tumor microenvironment, such as increased hypoxia and acidosis [1], a cascade of events takes place, resulting in the production of angiogenic "promoters" (stimulators) and a decrease in angiogenesis inhibitors [2–4]. One of the best known angiogenic stimulators, produced by most tumors, is the vascular endothelial growth factor (VEGF-A).

The VEGF-A gene undergoes alternative splicing between exons 5 and 8 (Fig. 1) [5]. This mechanism results in the predominant production of four isoforms, which differ in molecular weight, and are thus known as VEGF121, 165, 189, and 145. VEGF165 is the predominant secreted isoform, produced by most cell types (and most tumors), and although it is a diffusible protein, a significant fraction binds to the extracellular matrix (ECM). In contrast, VEGF121 is a freely diffusible isoform that does not bind to heparin, while VEGF189 binds strongly to heparin and therefore is completely sequestered in the ECM. The isoform 145 of VEGF is observed preferentially in carcinomas of the female reproductive system [6]. The importance of selective VEGF isoform secretion by tumors has been demonstrated [7]. In detail, tumors that secrete predominantly the VEGF121 isoform have increased dilated and peripheral blood vessels, while tumors overexpressing VEGF189, had highly branched and internal neo-vasculature [8]. More recently, increased ratio of VEGF121 versus the 165 or 189 isoforms was shown to be critical for the angiogenic phenotype of prostate cancers [9].

VEGF-A is produced by cells under stress, such as during hypoxia, resulting in tissue angiogenesis and oxygenation, although the molecular mechanisms regulating VEGF production in response to microenvironmental stimuli other than hypoxia, such as acidosis, are still poorly characterized [10].

Alternative splicing is a major mechanism for modulating the expression of cellular and viral genes and enables a single gene to increase its coding capacity. The VEGF isoforms mentioned above represent one family of proteins whose expression may be regulated by alternative splicing.

The family of SR (serine/arginine-rich) proteins has been implicated in splicing; they are characterized by an RNA recognition motif (RRM) and a C-terminal domain rich in alternating serine and arginine residues (the RS domain) [11]. The RRMs determine RNA binding specificity, whereas the RS domain mediates protein-protein interactions that are thought to be essential for the recruitment of the splicing apparatus and for the splice site pairing.

In the present report, we studied the influence of microenvironment cues that could affect the VEGF-A gene splicing pattern, and determined the molecular mechanisms involved.

Results

Microenvironment Changes Affect VEGF Alternative Splicing Pattern

We investigated how changes in the microenvironment might affect the pattern of VEGF alternative splicing (Fig. 1), using endometrial carcinoma cells as a model

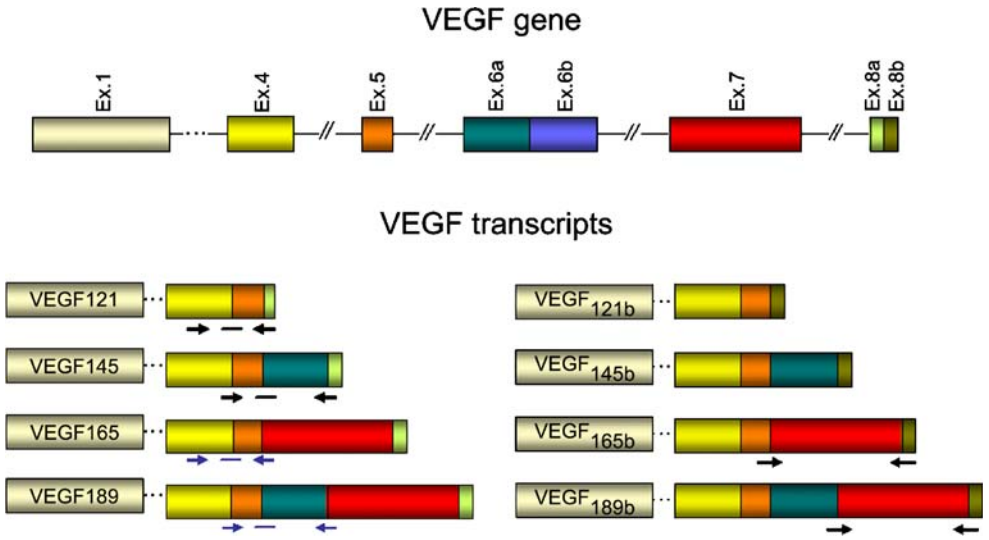

Fig. 1 VEGF isoforms that result from pre-mRNA alternative splicing. These isoforms differ in the presence or absence of exons 6 and 7 that codes for heparin-binding domains and in the presence of exon 8a or 8b that induce antagonist effects of VEGF. The localization of the probes and primers used to amplify the different isoforms of VEGF are indicated by a *line* or an *arrow*, respectively. The *lines* and *arrows in blue* represent the probes and primers used to amplify either VEGF165+VEGF165b or VEGF189+$VEGF_{189b}$

(since these cells express all VEGF-A isoforms). For this purpose, we induced changes in the culture medium (by exposing the cells to acidic pH, progesterone, β-estradiol, glucose and cobalt chloride, to mimic for hypoxia), and quantified the ratio of VEGF isoforms by real time RT-PCR (RQ-PCR). As expected, hypoxia significantly increased VEGF production, as did acidosis (Fig. 2a,b and Supplementary Fig. 1). However, a more evident shift in the pattern of VEGF isoforms produced, occurred in samples subjected to lower pH. A pH 5.5 induced a preferential VEGF121 increase ($p<0.001$), suggesting it modulated the mechanisms involved in alternative splicing.

The $VEGF_{xxx}b$ isoforms, previously shown to be involved in the regulation of tumor angiogenesis [12], were expressed at very low levels by the cell line used in our studies. Notably, in this study $VEGF_{xxx}b$ isoforms did not accompany the variations observed in VEGFxxx isoforms ($VEGF_{165}b$ and $VEGF_{189}b$ did not differ significantly from control to pH 5.5 conditions, $p>0.05$). Therefore we concluded that the modifications in VEGF alternative splicing observed in low pH, did not involve the $VEGF_{xxx}b$ alternative transcripts (Fig. 3).

The shift in VEGF isoform ratio occurred within 6 h of pH 5.5, but the greatest difference was seen after 8 h of stimulation (Fig. 4a).

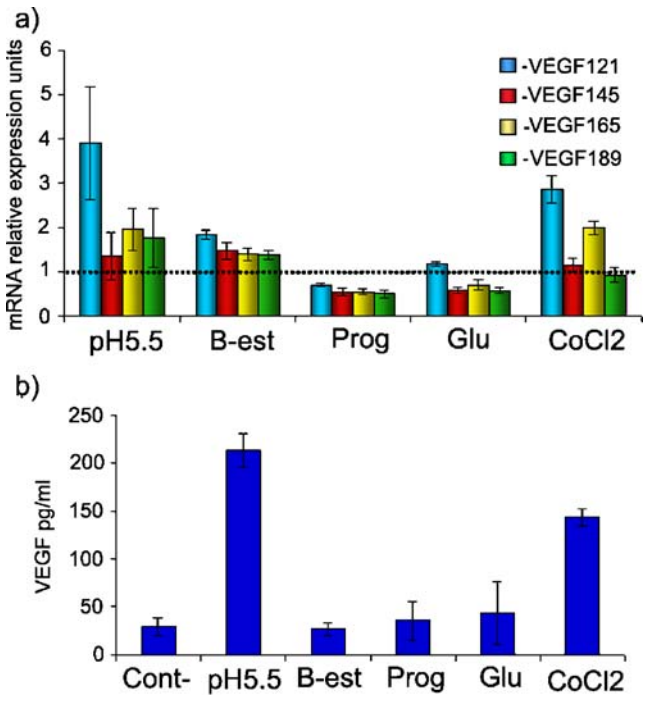

Fig. 2 VEGF isoforms expression pattern by RL95 cells in response to changes in the microenvironment. By real time RT-PCR (**a**) and ELISA (**b**), we can see an increase in VEGF production in acidic and hypoxic (mimicked by $CoCl_2$) conditions. A shift in the VEGF isoforms splicing pattern is more evident at pH 5.5 where VEGF121 expression is significantly different from all other isoforms ($p<0.001$). In hypoxic conditions VEGF121 is also significantly different from VEGF145 and 189 ($p<0.001$) and from VEGF165 ($p<0.05$). In **a** the level of each isoform in different conditions is represented relatively to control conditions (which is considered equal to 1). In graph **a** $n=10$ and in graph **b** $n=3$

VEGF Isoform Shift in Acidic pH is Accompanied by p38/MAPK Activation

Since acidic pH induced a shift in isoform production by RL95 cells, next we sought to define the signaling pathways that might be involved in this effect.

As shown in Fig. 4b, 8 h in acidic pH induced the activation of the stress signaling pathways p38 MAPK ($p<0.05$) and SAPK/JNK, while ERK and Akt remained unchanged. In vitro blockade of the two signaling pathways using specific inhibitors demonstrated that cells cultured in the presence of the p38 pathway inhibitor (SB202190) did not respond to the acidic pH (Fig. 4c). Under these conditions the shift in VEGF isoform production was not observed (VEGF121 was not significantly different from all the other isoforms, $p>0.05$), while the SAPK/JNK inhibitor SP600125 had little effect (VEGF121 vs VEGF165, $p<0.001$, as observed in pH 5.5 condition). These data indicate that the p38 stress signaling pathway may be involved in the effects of acidic pH that result in modulation of the VEGF alternative splicing pattern.

SR Proteins Could be Involved in the Regulation of VEGF Isoforms

SR proteins have been described to be involved in the control of constitutive and alternative splicing of genes [11]. To evaluate which SR proteins could be activated by the p38 stress signaling pathway and involved in VEGF isoform shift in acidic conditions, the modulation of SR proteins was investigated.

Using an antibody against phosphorylated-SR proteins, we verified that the up-regulation of these proteins correlates with activation of the p38 signaling pathway and the shift in VEGF isoform production (Fig. 5a). Since these proteins can shuttle between the nucleus and cytoplasm we confirmed that the majority of these proteins were localized at the nucleus in pH 5.5 (data not shown) and could be acting as splicing regulators.

By real time RT-PCR we quantified the mRNA of different SR proteins (SF2/ASF, SRp20 and SRp40) and observed that pH 5.5 induced a significant up-regulation ($p<0.05$) of SRp20 that could be partially inhibited in the presence of SB202190, the p38 MAPK inhibitor (Fig. 5b). This result indicates that p38 MAPK was not acting at the SR proteins mRNA levels and could have a role at mRNA translation or SR proteins activation (protein phosphoryla-

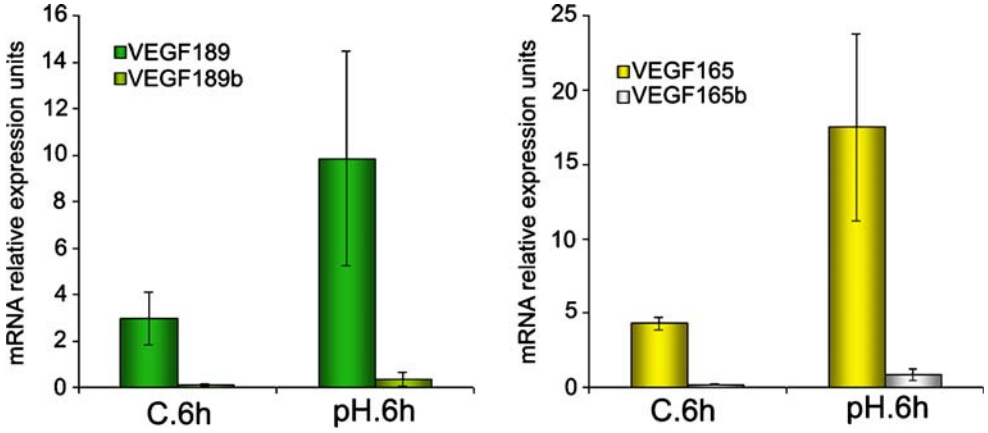

Fig. 3 Discrimination between VEGFxxx and $VEGF_{xxx}b$ isoforms expression in acidic pH. In pH 5.5 there was a significant increase in VEGF165 ($p<0.001$) and 189 ($p<0.01$) but the expression of VEGF165b ($p<0.001$) and 189b ($p<0.001$) is too low when compared respectively to the VEGF165 and 189 and do not change in acidic conditions ($p>0.05$ for both isoforms). The graphs represent the mean of three experiments

tion). To reveal the involvement of selective SR proteins in VEGF alternative splicing we used siRNA against each SR protein and tested the effect in the pattern of VEGF isoform production. siRNA against SF2/ASF, SRp20 or SRp40 reduced the production of the VEGF121 observed in pH 5.5 (in fact, the VEGF isoforms pattern in these conditions is equal to the control), suggesting that not only we have prevented VEGF expression but also have prevented the shift in the VEGF isoforms pattern ($p>0.05$; Fig. 6b). Thus, SF2/ASF, SRp20 and SRp40 may have a role at the VEGF alternative splicing process. These three proteins might also have a role in the alternative slicing of a VEGF regulator since by its down-regulation we affected the VEGF expression.

Since these proteins have RNA recognition motifs, we used bioinformatics software to search putative binding

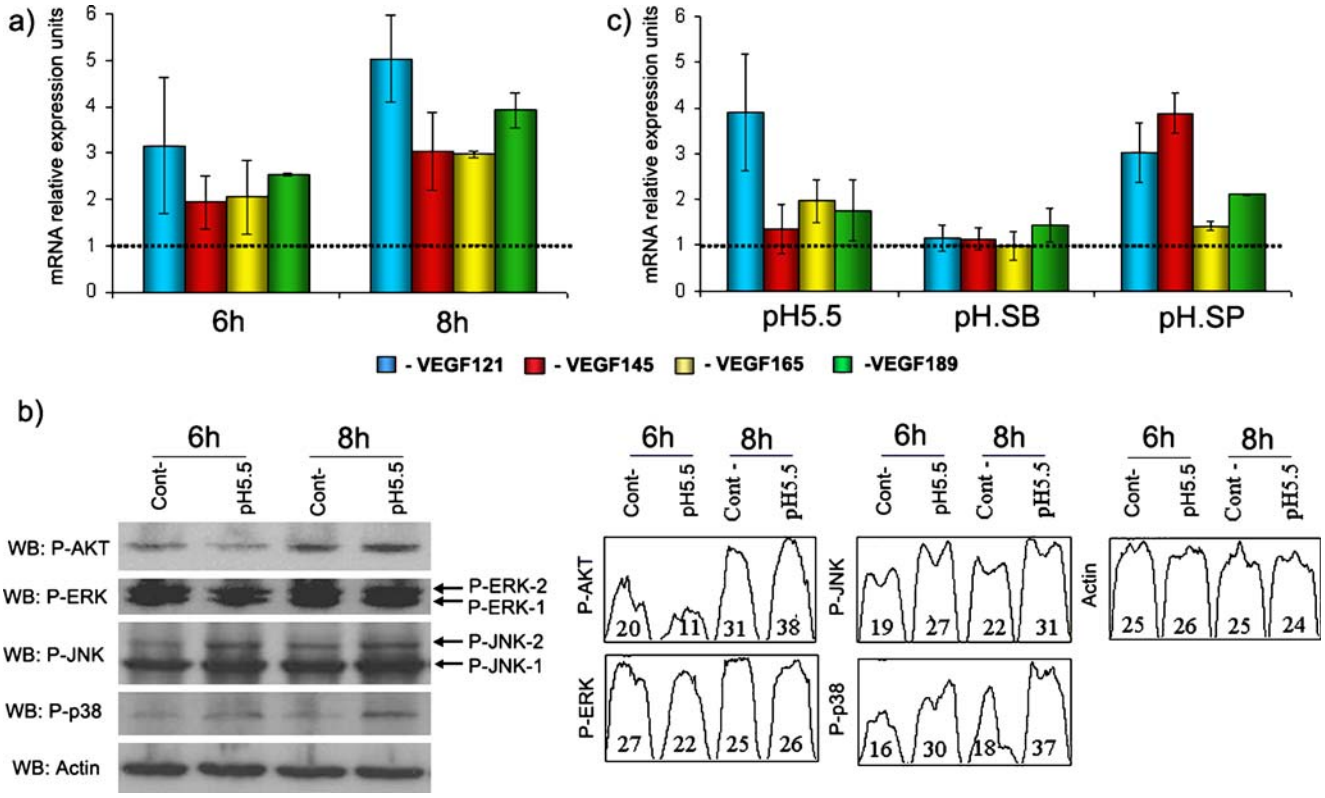

Fig. 4 **a** Correlation between VEGF isoforms pattern inversion and the activation of stress signaling pathways in acidic conditions. After 8 h of pH 5.5 stimulation, the changes in VEGF isoform splicing pattern are more pronounced. **b** As shown by western blotting and densitometry quantification, at this time point, from the several signaling pathways analyzed, only the p38 MAPK ($p<0.05$) and the SAPK/JNK stress pathways were activated. **c** SB202190 and SP600125 were used to inhibit p38 MAPK and SAPK/JNK stress pathways, respectively. In the presence of the p38 inhibitor, low pH failed to induce a shift in VEGF splicing pattern (all VEGF isoforrms are similar, $p>0.05$), while the SAPK/JNK inhibitor could not prevent the shift in VEGF isoforms (VEGF121 is significantly different from VEGF165, $p<0.001$). Graphs **a** and **c** represent the mean of three experiments and the blot was repeated three times

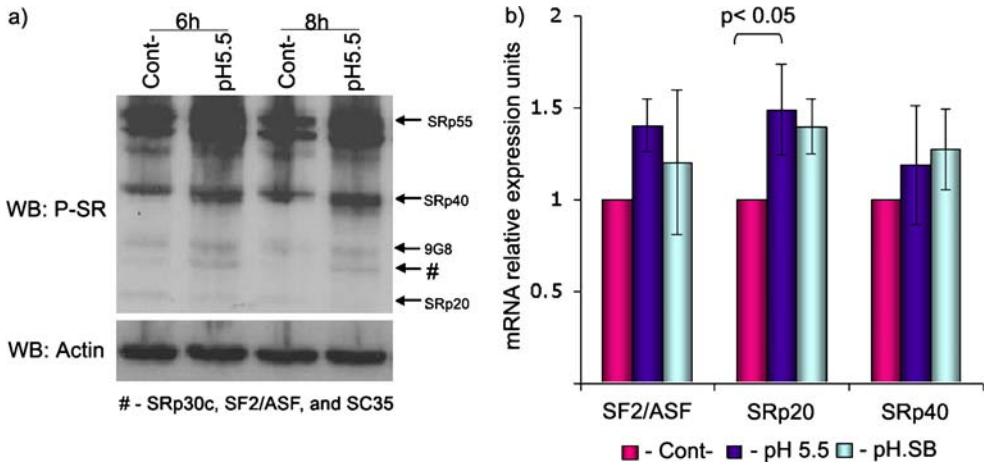

Fig. 5 **a** SR proteins involved in the control of the VEGF splicing pattern. Most SR proteins were up-regulated in acidic conditions both after 6 and 8 h of exposure. **b** Expression of the SR protein SRp20, in RL95, increased significantly ($p<0.05$) in acidic conditions and was only partially inhibited in the presence of the p38 MAPK inhibitor SB202190. In graph **b** the *error bars* represent the standard deviation of three independent experiments

sites for these specific SR proteins, SF2/ASF, SRp20 and SRp40. This approach showed that SRp20 and SRp40 do not have putative binding sites at the exons of VEGF that are involved in alternative splicing, but SF2/ASF has putative binding sites at exons 5, 7 and 8 of VEGF. Therefore, we may speculate that the SF2/ASF protein may act on alternative splicing of VEGF by binding directly to the VEGF sequence and in contrary, the SRp20 and SRp40 could be exon-binding independent by recruiting splicing factors through its serine/arginine rich domain (SR proteins property already described by Wu and Maniatis in 1993) [13].

Since SR proteins are large families of proteins involved in the global mechanisms of alternative splicing, a more detailed study must be done in order to identify other SR or hnRNP (SR proteins antagonists, heterogeneous nuclear ribonucleoproteins) proteins that may regulate the VEGF isoforms pattern in acidic conditions.

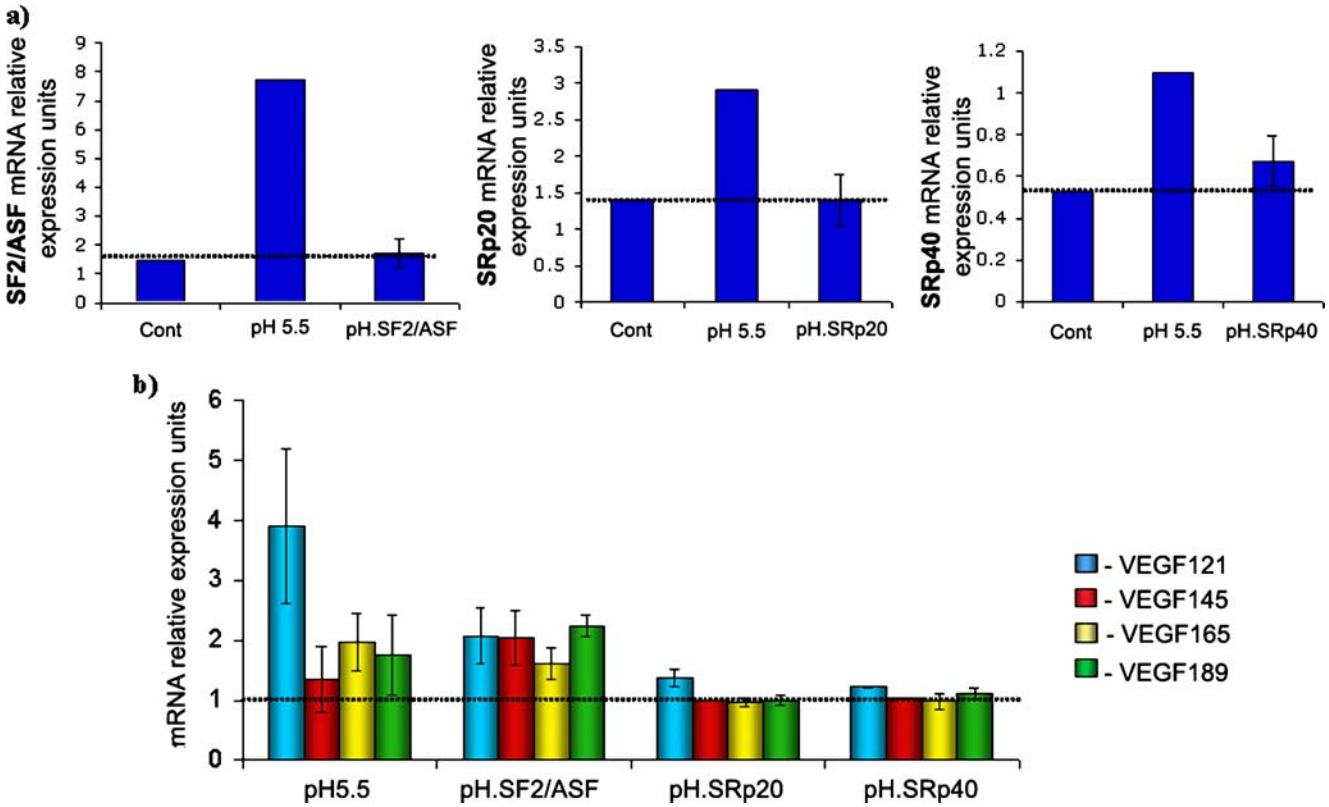

Fig. 6 **a** Effect of SR proteins down-regulation in VEGF alternative splicing in acidic conditions. siRNA for SF2/ASF, SRp20 and SRp40 (performed in triplicate) were used and its efficacy was demonstrated by real time RT-PCR. **b** Using siRNA for SF2/ASF, SRp20 and SRp40, there was no shift in the VEGF isoforms pattern observed in acidic conditions, since all isoforms were similar to each other ($p>0.05$) and to the control ($p>0.05$)

Taken together, we propose a model where the activation of the p38 stress signaling pathway in response to a microenvironment signal (such as a decrease in pH levels) accompanies a change in VEGF alternative splicing and an increase in alternative splicing factors phosphorylation (SR proteins; see our proposed model in Fig. 7).

Discussion

Angiogenesis is an essential property of all tumors, allowing tumor expansion and contributing towards metastasis dissemination. Since it is a common feature of most malignancies, the importance of angiogenesis as a therapeutic target has been well documented [14]. Regarding the molecular signals that control the tumor angiogenesis process, the increased production of pro-angiogenic factors by most tumors has received great attention, most notably the recognition of VEGF-A as a key angiogenic factor for the majority of tumors [15]. VEGF-A has several isoforms, whose importance in the context of tumor angiogenesis has already been addressed. Although VEGF-A isoforms can bind differently to the ECM, they have also been attributed with different capacity to modulate the tumor vasculature [8, 16]. In detail, VEGF 121 has been described as more angiogenic and tumorigenic in breast [17] and prostate [9] cancer and also to specifically improve oxygenation in experimental breast tumors [18], while VEGF189 contributes to the establishment of distant metastasis of pulmonary adenocarcinoma [19]. Given the lack of mechanistic information concerning the regulation of the VEGF alternative splicing process, in the present report we hypothesized that cues in the tumor microenvironment might selectively affect the VEGF splicing pattern, and studied the mechanisms involved in this effect.

Tumor microenvironment signals such as hypoxia and acidosis have been suggested to play a major role in the control of VEGF-A production, and consequently in modulation of angiogenesis [20]. In fact, hypoxia and acidosis are common features of the majority of solid and hematologic malignancies, perhaps as a consequence of the tumor metabolic needs, or as a consequence of an altered (pro-malignant) microenvironment. Importantly, the extracellular pH has been recognized as an inducer of VEGF, and also to regulate the VEGF interactions with different cells and with components of the extracellular matrix [21].

In the present study, acidosis consistently affected the VEGF alternative splicing pattern produced by endometrial cancer cells (used as a model); this correlated to a shift in the usual VEGF isoform expression pattern, resulting in an significant increase in the VEGF121, that was not accompanied by the other VEGF isoforms.

The involvement of signaling pathways and splicing machinery had not been studied in the context of VEGF alternative splicing. In our report, we reveal the involvement of p38 and possible members of the SR protein family in this mechanism. We now intend to perform a more detailed characterization of the splicing machinery involved in the VEGF alternative splicing in tumor cells exposed to different microenvironment cues.

Importantly, in addition to alternative splicing regulation, the levels of each isoform of VEGF can also be modulated

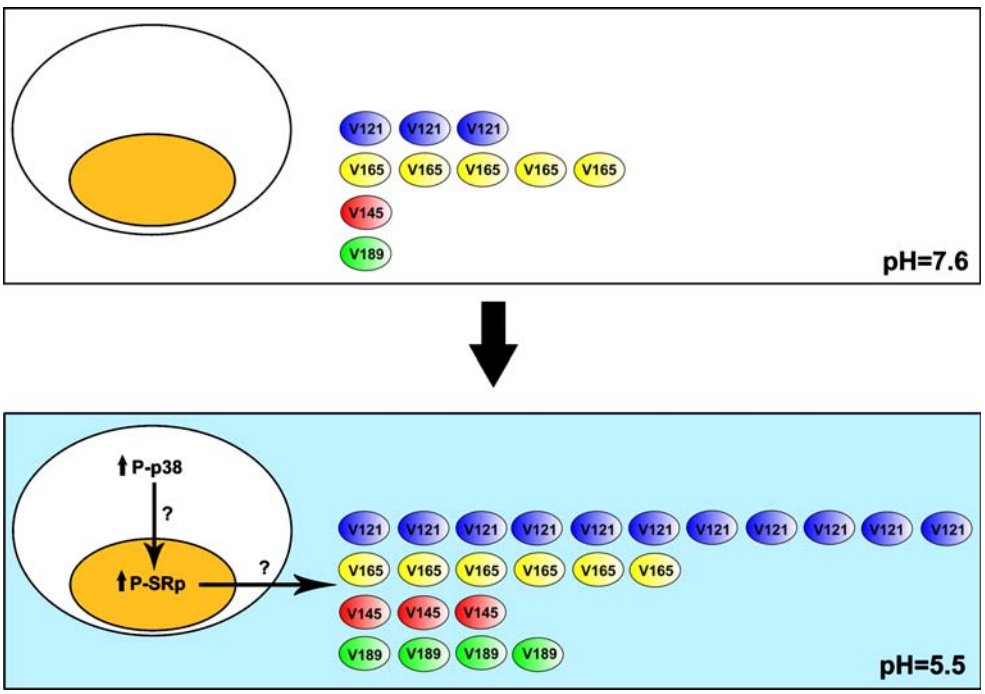

Fig. 7 Proposed model. When endometrial cancer cells are exposed to acidic medium the p38 MAPK signaling pathway is activated and an increase in SR proteins phosphorylation that will eventually regulate the VEGF alternative splicing takes place. In acidosis, not only VEGF expression is increased but also a modification of the alternative splicing occurs, where an increase in the proportion of VEGF121 versus VEGF145, 165, and 189 is observed

Table 1 Primers and probes used in real time RT-PCR for VEGF isoforms

VEGF isoforms	Probes (6-FAM-5′ → 3′-TAMRA)	Primers (5′ → 3′)	
121	ACAGCACAACAAATGTGAATG CAGACCAAA	Forward	CCAGCACATAGGAGAGATGAGCTT
		Reverse	CGGCTTGTCACATTTTTCTTGTC
145	AGAGCAAGACAAGAAAAAAAA TCAGTTCGAGGAA	Forward	AATGTGAATGCAGACCAAAGAAAG
		Reverse	CACATACGCTCCAGGACTTATACC
165+165b	ACAGCACAACAAATGTGAATG CAGACCAAA	Forward	CCAGCACATAGGAGAGATGAGCTT
		Reverse	AGGCCCACAGGGATTTTCTT
189+189b	AGAGCAAGACAAGAAAAAAAA TCAGTTCGAGGAA	Forward	AATGTGAATGCAGACCAAAGAAAG
		Reverse	AGGGAACGCTCCAGGACTTATA
165b	–	Forward	AAGAAAATCCCTGTGGGCCTT
		Reverse	TGGTGAGAGATCTGCAAGTACGTT
189b	–	Forward	CTGGAGCGTTCCCTGTGG
		Reverse	TGGTGAGAGATCTGCAAGTACGTT

at the mRNA stability level. Pagès et al. [22] demonstrated that anisomycin (a strong activator of stress-activated protein kinases, SAPKs) increased VEGF mRNA stabilization through the activation of p38 kinase and JNK. This protein induce the recruitment of HuR (one of the Hu family proteins) and PAIP2 (poly(A)-binding protein-interacting protein 2) to the AU-rich elements (AREs) in the 3′-untranslated region (3′ UTR) of VEGF mRNA [23]. Since the ARE sequence at the 3′ UTR of the VEGF mRNA is present in all the isoforms, there is a possibility that all isoforms have the same stability and that the differences observed in VEGF isoforms ratio were not due to a difference in mRNA stability. In fact, a study that correlates the VEGF144 up-regulation with glucose starvation, suggest that other mechanism apart mRNA stability must also exist to explain the dramatic increase observed (the increased stability observed for this isoform was approximately threefold but the increase in mRNA was ~400-fold) [24]. Nevertheless, more studies have to be done to address this important question.

Additionally, the p38 pathway, which is an important stress signaling pathway that have been described to control VEGF at the mRNA expression [25] and stability level [22], was also shown to act directly or indirectly in the alternative splicing of this gene.

In conclusion, we postulate that changes of VEGF isoforms observed in acidic conditions may represent the adaptation of tumors to alterations in the microenvironment, namely by activating angiogenic signaling pathways, through different VEGF isoforms production.

Material and Methods

All reagents were obtained from Sigma, unless otherwise stated.

Cell Lines and Cell Culture Conditions

The RL95 cell line was kindly provided by Professor Steve Smith (currently Principal of the Faculty of Medicine, Imperial College, London, UK). It was cultured in 50% high glucose Dulbecco's modified Eagle's medium (DMEM) medium (Sigma) and 50% nutrient mixture F-12 Ham (Sigma), supplemented with 10% FBS, 100 μg/ml of streptomycin sulfate, 100 U/ml of penicillin G sodium, 2 mM of L-glutamine and 0.1 μg/ml of amphotericin B as Fungizone. Cells were cultured at 37°C in a 5% CO_2 atmosphere.

Upon reaching confluency, RL95 cells were submitted to changes in pH (pH 5.5, to mimic for acidosis), oxygen, glucose (100 mM) or hormones levels. To obtain an acidic medium we used HCl and the pH 5.5 was confirmed before and after the experiment using a pH electrode. To induce hormonal changes and to mimic hypoxia, β-estradiol (100 nM)/progesterone (1 μM) and cobalt chloride (150 μM) [26] were used respectively.

To test the importance of the different signaling pathways in the regulation of VEGF alternative splicing, the RL95 cell line was cultured in growth medium at pH 5.5 with or without the inhibitors of p38 MAPK (SB202190, Sigma, 20 μM,) and SAPK/JNK (SP600125, Sigma, 20 μM) signaling pathways.

RNA Isolation and Real Time RT-PCR with TAQMAN or Sybergreen

RNA was extracted by Trizol reagent (Invitrogen) and 2 μg of total RNA was used to synthesize cDNA [27]. The mRNA level of each VEGF isoform was measured by real time RT-PCR (TAQMAN or Sybergreen) on the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems) using specific primers and probes represented

in Fig. 1 and Table 1. Expression of different SR proteins was analyzed with Sybergreen with specific primers (SF2/ASF: forward—5′-GAA CAA CGA TTG CCG CAT CT-3′, reverse—5′-AAT CAT AGC CGT CGC GAC C-3′; SRp20: forward—5′-GCA TCG TGA TTC CTG TCC ATT-3′, reverse—5′-GTT CTT CCA TCT AGC TCT CGG ACT-3′; SRp40: forward—5′-CTG TCG GGT ATT CAT CGG GA-3′, reverse—5′-ACG GTC AGA GTA TCG TCC TCT ACC T-3′). Real time PCR program consisted of an initial denaturation step at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The housekeeping gene used to normalize the samples in TAQMAN assay was the 18S (Human 18S rRNA—20×, Applied Biosystems). For Sybergreen, the BCR gene was used (forward—5′-GAG CGT GCA GAG TGG AGG GAG AAC A-3′; reverse—5′-CAC AGT ATC CTC AGG GTC TGG GA-3′).

The relative expression of each sample was calculated with respect to a standard calibration curve that represents a serial dilution of a cDNA. Each sample was analyzed in triplicate and each PCR experiment included at least one non-template control well.

ELISA, Protein Extraction and Western Blotting

Culture supernatants from RL95 in different conditions were collected and used to measure human VEGF by ELISA (Oncogene Research Products) under conditions described by the supplier.

To extract total proteins the pellets were suspended in a buffer containing 1% NP40, 10% glycerol, 50 mM Tris–HCl pH 7.5, 0.1% sodic azid and 150 mM NaCl, supplemented with protease and phosphatase inhibitors. After 30 min in ice, lysates were centrifuged for 15 min at 4°C and 12,000 rpm.

Equal proteins amount were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blots were incubated overnight with an antibody against phosphorilated SR proteins [mouse anti-SR proteins 1 (H4) from Zymed] at a concentration of 10 μg/ml. Antibodies against P-AKT, P-ERK, P-JNK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), P-p38 (cell signaling) and actin (Sigma) were used at final concentration of 100 ng/ml, 400 ng/ml, and a dilution of 1:500, 1:1,000 and 1:2,000 respectively. Western blotting was performed following conventional protocols. Blots were revealed with the ECL (Amersham) system, according to manufacturers instructions. The hybridizing signals were quantified with ImageJ software.

siRNA for SR proteins

One day before transfection, 1×10^5 RL95 cells were plated in 500 μl of growth medium without antibiotics in a 24-well culture vessel. Fifty picomoles of each siRNA (SFRS1 for SF2/ASF; SFRS3 for SRp20 and SFRS5 for SRp40 from Ambiom Company) were used. The RNA and 1 μl of Lipofectamine 2000 (Invitrogen) were diluted separately in 50 μl of Opti-MEM I reduced serum medium. After 5 min of incubation, the two dilutions were combined and incubated for 20 min at room temperature. This mixture was then added to the cells following an incubation of 6 h at 37°C and 5% CO_2. After transfection, the medium was replaced. The effect of transfection was assessed after 72 h.

Bioinformatics Approach/Program

The splicing rainbow (Morais & Valcarcel EMBL 2002 at http://www.ebi.ac.uk/asd-srv/wb.cgi?method=8) bioinformatics software was used to find putative binding sites for SF2/ASF, SRp20 and SRp40 proteins in the VEGF sequence. From the list of binding sites received after the VEGF sequence analysis we only choose as putative binding sites the sequences with higher scores (S) that were in exons. In detail, a sequence was considered a putative binding site for SRp20, and SF2/ASF if $S>6$ and for SRp40 if $S>5$.

Statistical Analysis

Results are expressed as mean ± standard deviation. Data were analyzed using the unpaired two-tailed Student's t test or the one-way ANOVA with post Tukey test. p values of <0.05 were considered significant.

Acknowledgement We are grateful to Nuno Morais (PhD student, Unidade de Biologia Celular, Instituto de Medicina Molecular, Lisbon, Portugal) for his help in the bioinformatics analysis. We also thank Professor Steve Smith (currently Principal of the Faculty of Medicine, Imperial College, London, UK) for providing the RL95 cell line, and Mr. Alex Varey (Microvascular Research Laboratories, University of Bristol) for his useful suggestions regarding the $VEGF_{xxx}b$ isoforms. Ana Paula Elias is a recipient of SFRH/BD/14287/2003 Fellowship (from the Portuguese Foundation for Science and Technology, FCT). This study was supported by POCTI 38391/2001 (Sérgio Dias) and by Liga Portuguesa Contra o Cancro, Nucleo Regional Sul.

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