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Advances in Brief

Relation of Hypoxia-inducible Factor-2α (HIF-2α) Expression in Tumor-infiltrative Macrophages to Tumor Angiogenesis and the Oxidative Thymidine Phosphorylase Pathway in Human Breast Cancer

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Abstract

Tumor-associated macrophages (TAMs) produce angiogenic factors and in breast cancer are associated with high vascular grade and poor survival. TAMs preferentially migrate to hypoxic areas within tumors and strongly express hypoxia-inducible factor (HIF)-2α. This study examined whether HIF-2α was involved in TAM angiogenic activation by correlating its expression with tumor microvessel density as a marker of angiogenesis, and other tumor variables, in a series of human primary invasive breast carcinomas. A correlation was found between high TAM HIF-2α and high tumor vascularity ($P < 0.0001$), as well as high tumor grade ($P = 0.007$). The relation of HIF-2α expression to a recently described oxygen-dependent pathway of angiogenesis was also studied, and an inverse relationship was found between TAM HIF-2α and tumor thymidine phosphorylase expression ($P = 0.02$). These results suggest that TAM HIF-2 signaling may be a useful target for future antiangiogenic strategies but show that tumors use both oxygen-dependent and oxygen deficiency-regulated pathways for angiogenesis. Thus, combined blockade of pathways and careful assessment of these pathways in trials are necessary.

Introduction

It is well established that TAMs[2] are major contributors to angiogenesis. They secrete angiogenic cytokines, such as VEGF and tumor necrosis factor-α (1). Additionally, macrophages express high levels of an enzyme that influences angiogenesis, TP (platelet-derived endothelial cell growth factor), and other extracellular matrix-degrading enzymes, such as metalloproteases and collagenase, which release cytokines sequestered by the extracellular matrix (1). We have shown previously that increased focal infiltration of macrophages into human breast tumors is directly associated with both angiogenesis and poor relapse free and OS (2). We have also shown that increased levels of necrosis, a feature of aggressive hypoxic tumors, correlate with high TAM indices and increased angiogenesis (3).

Hypoxia is important in tumor progression and has been correlated with various indicators of tumor aggression, as well as metastasis and poor prognosis in a number of tumor types (4). The homologous transcription factors, HIF-1 and HIF-2, are both members of a family of transcription factors termed bHLH/PAS proteins, which mediate the hypoxic regulation of a number of specific genes via their interaction with hypoxia response elements (5, 6). In a study of HIF-1α and HIF-2α in different tumor types, we found that HIF-2α surprisingly was commonly up-regulated in stromal cells rather than tumor cells (7). These cells were TAMs, and normal tissue macrophages did not express HIF-2α, which is a major phenotypic difference between tumor and normal macrophages. Therefore, we quantified the extent of HIF-2α expression in the TAM population of a series of invasive human breast carcinomas and compared this to the degree of angiogenesis in these tumors and other markers of tumor aggression, as well as RFS and OS.

We have shown previously that TP is markedly up-regulated in a subset of breast tumors (8) and that one mechanism by which it induces angiogenesis is via oxidative stress (9). Previously, we have also found great heterogeneity in the expression of angiogenic growth factors in breast cancer (10), and one mechanism may relate to differences in oxygenation. Therefore, we also assessed whether there were *in vivo* differences in these two major pathways that have divergent interactions with hypoxia.

Materials and Methods

Tumors and Patients. A consecutive series of 139 surgically resected invasive breast tumors were retrieved from the archives of the John Radcliffe Hospital, Oxford, United Kingdom, the clinical and pathological details of which are shown in Table 1. All had level 1 axillary node dissection, and the presence of nodal metastasis was confirmed histologically. Invasive carcinomas of ductal type were graded using the modified Bloom and Richardson method, and all patients were followed up every 3 months for the first 18 months and every 6 months thereafter. All patients received either simple mastectomy or lumpectomy and radiotherapy; adjuvant radiotherapy was administered to the ipsilateral axilla if histological evidence of nodal metastasis was found. Patients with confirmed recurrent disease were treated by endocrine manipulation for soft tissue or skeletal disease or by chemotherapy for visceral disease or failed endocrine therapy. Patients with isolated soft tissue relapse received radiotherapy. Adjuvant treatment consisted of Tamoxifen for 5 years for ER-positive premenopausal and postmenopausal patients and cyclophosphamide, methotrexate, and 5-fluorouracil i.v. for six courses for node-positive premenopausal and ER-negative, node-positive postmenopausal patients (Table 1).

HIF-2α Immunohistochemistry. Staining was performed on paraffin-embedded and formalin-fixed 5-μm sections. After dewaxing and rehydration, endogenous peroxidase was blocked in a 0.5% hydrogen peroxide in water solution for 30 min. Antigen retrieval involved pressure cooking in 50 mmol Tris/0.2 mmol EDTA buffer for 180 s or incubation at 60°C for 16 h in the same buffer. The previously categorized and validated monoclonal antibody 190b against HIF-2α (7) was used as the primary antibody at a concentration of 15 μg/ml and incubated on the histological sections for 90 min at room temperature. Negative controls involving omission of the primary antibody were included with each staining run. Staining was amplified using a secondary horseradish peroxidase-conjugated goat antimouse serum antibody

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[2] The abbreviations used are: TAM, tumor-associated macrophage; VEGF, vascular endothelial growth factor; TP, thymidine phosphorylase; CVC, Chalkley vascular count; HIF, hypoxia-inducible factor; EGFR, epidermal growth factor receptor; ER, estrogen receptor; OS, overall survival; RFS, relapse free survival.

Table 1 *Summary of clinicopathological features of patients and tumors*

Patient characteristics	No.
Age	
Median (range) years	57 (28–82)
<50	44
≥50	95
Surgical treatment	
Lumpectomy + RT[a]	100
Mastectomy	39
Adjuvant treatment	
Chemotherapy	36
Tamoxifen	103
Lymph node status neg/pos	71/68
Tumor size	
Median (range) cm	2.4 (1–8)
<2	40
≥2	99
Histology	
Ductal	124
Lobular	10
Others	5
Grade (ductal carcinomas only)	
I	17
II	53
III	49
ER[a]	
Median (range)	27.3 (0–742)
<10	53
≥10	86
EGFR[a]	
Median (range)	19 (0–212)
<20	70
≥20	69
Survival follow-up	
Median (range) months	83 (5–126)
Deaths, recurrences	43, 54

[a] fmol/mg^{-1} protein.
RT, radio therapy.

(DAKO, Ely, United Kingdom) at a dilution of 1/200 incubated for 30 min. This was followed by further incubation with a horseradish peroxidase-conjugated rabbit antigoat serum antibody (DAKO) at a dilution of 1/100 for 30 min. Staining was visualized with diamino-benzidine yielding a dark brown reaction product. After washing in pure water, the slide preparations were aqueously mounted (Aquamount; BDH, Poole, United Kingdom).

Quantification of TAM HIF-2α Staining. The number of HIF-2α-positive focal macrophage clusters was counted microscopically across an entire representative section of each invasive tumor at a magnification of ×10. The number of microscopic fields containing tumor was recorded simultaneously. After calibration of a ×10 magnification field to mm^2, the number of HIF-2α-positive TAM hotspots/mm^2 could be calculated. For the purpose of cutpoint analysis, a value of 0.07 HIF-2α-positive TAM hotspots/mm^2 (corresponding to the mean) was used to separate tumors into high and low groups. A TAM hotspot was defined as a morphologically distinct and focal cluster of TAMs. On some occasions, adjacent hotspots were connected by an attenuated ribbon of intervening TAMs. Such hotspots were considered for the purpose of this study to be distinct enough to be enumerated separately (Fig. 1).

Immunohistochemistry of TP and Blood Vessels (Vascular Grading). TP immunohistochemistry had been assessed previously on this series of cases using the monoclonal antibody PG44c. A standard streptavidin peroxidase method with diamino-benzidine was then used to visualize staining. Tumors were considered positive if >25% of the neoplastic cells displayed moderate staining for TP (Fig. 1; Ref. 8). Vessels were identified using either the monoclonal antibody JC70a (against CD31) or QBEnd10 (against CD34) after enzymatic antigen retrieval and standard visualization using methods described above. CVC was determined quantitatively using the Chalkley point counting method also described previously (2). This involved assessment of the three most vascular areas (hot spots) at ×25 magnification. A CVC ≥ 7 (corresponding to the upper tertile) defined high and low groups for cutpoint analysis.

ER and EGFR. ER analysis was performed by ELISA (Abbott Laboratories, Maidenhead, UK). Cytoplasmic oestrogen levels > 10 fmol/mg protein were considered positive. EGFR was determined using ligand binding of (^{125}I)-EGF to tumor membranes. EGFR levels > 20 fmol/mg protein were considered positive.

Statistical Analysis. χ^2 tests examined relationships between categorical tumor variables. Where the number of categories was two, Mann-Whitney nonparametric tests were used to compare categorical with continuous tumor variables. Where the number of categories was greater than two, Kruskall-Wallis nonparametric tests were used. Spearman-rank correlations were used to investigate relationships between continuous patient and tumor variables. These comparative analyses were performed using Statview 4.5 statistical analysis software (Abacus Concepts, Inc., Berkeley, CA). Survival curves were plotted using the method of Kaplan and Meier, and the Log-rank test and Cox univariate duration model were used to evaluate differences between life tables. A Cox multivariate proportional hazard model was used to investigate the overall effect of patient and tumor variables on survival. These analyses were performed using Stata release 3.1 (Stata Corp., College Station, TX).

Results

TAM HIF-2α Expression and Tumor Angiogenesis. A group of 139 cases was stained for HIF-2α, and of these, 45 exhibited some immunoreactivity for HIF-2α (35%). In this series of cases, the number of HIF-2α-positive macrophage hotspots/mm^2 ranged from 0 to 1.03 with a mean value of 0.07, which was used as the cutpoint for categorical analysis. A group of 128 of the 139 cases was also stained successfully for blood vessels. A group of 47 fell into the high vessel grade category (37%) using the standard cutpoint of 7. In this series, vessel counts ranged from 2.67 to 11.33 with a mean value of 6.2. A positive correlation was found between increasing microvessel count (as a continuous variable) and increased numbers of HIF-2α-positive TAM hotspots (Spearman Rho = 0.38, $P < 0.0001$). After categorization using the cutpoints indicated above, a positive association was also seen when high and low categories for vessel grade and TAM HIF-2α expression were compared ($\chi^2 = 22.59$, $P < 0.0001$; Table 2). TAM HIF-2α expression was significantly greater in the high angiogenesis group *versus* the low angiogenesis group (Mann-Whitney $P < 0.0001$; Fig. 2*A*), and similarly, mean microvessel count was higher in the high *versus* low TAM HIF-2α-expressing groups (Mann-Whitney $P < 0.0001$).

TAM HIF-2α Expression and Clinical Tumor Variables. The degree of TAM HIF-2α expression was compared with other breast tumor variables. No associations were found between TAM HIF-2α and histological type, tumor size at excision, patient age, menopause

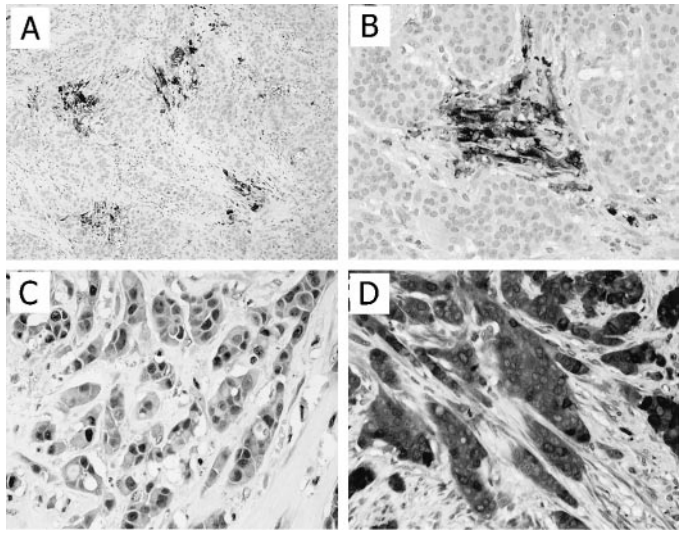

Fig. 1. HIF-2α staining in invasive ductal breast carcinoma. *A*, ×10 magnification view of four macrophage hotspot clusters in a tumor with high levels of HIF-2α-expressing macrophage clusters. *B*, ×25 magnification view of single HIF-2α-positive macrophage cluster in an invasive ductal breast carcinoma. *C*, ×25 magnification view of TP tumor staining in 25% of neoplastic cells. *D*, ×25 magnification view of TP tumor staining in 100% of neoplastic cells.

Table 2 *Multivariate Cox proportional hazards analysis for RFS and OS*

A. Overall survival

Variable	Hazard ratio	95% CI[a]	*P*
Size (≤2 cm/>2 cm)	2.3	1.04–5.08	0.04
Node status (neg/pos)	2.71	1.37–5.37	0.004
ER (neg/pos)	0.63	0.32–1.26	0.2
EGFR (neg/pos)	1.2	0.62–2.33	0.59
HIF-2α (<mean/≥mean)	1.97	0.97–4.00	0.06

B. Relapse survival

Variable	Hazard ratio	95% CI	*P*
Size (≤2 cm/>2 cm)	2.58	1.31–5.09	0.006
Node status (neg/pos)	2.42	1.35–4.35	0.003
ER (neg/pos)	0.52	0.29–0.95	0.03
EGFR (neg/pos)	0.87	0.49–1.53	0.63
HIF-2α (<mean/≥mean)	1.45	0.77–2.72	0.25

[a] CI, confidence interval.

status, or axillary node status. Grade was recorded in 119 of the 124 ductal carcinomas, and a marked association was noted between high TAM HIF-2α expression and increasing tumor grade ($\chi^2 = 9.99$, $P = 0.007$; Kruskall-Wallis $P = 0.002$; Fig. 2*B*). No correlations or associations were found between TAM HIF-2α expression and either ER or EGFR.

TAM HIF-2α Expression and TP. There was an inverse relationship observed between increased TAM HIF-2α expression and positive tumor TP expression analyzed either as a continuous variable or when categorized into high and low HIF-2α expression (Mann-Whitney $P = 0.0007$; $\chi^2 = 5.96$, $P = 0.015$; Fig. 2*C*).

TAM HIF-2α Expression and Survival. The degree of TAM HIF-2α expression was compared with RFS and OS. TAM HIF-2α expression was not associated with RFS ($P = 0.17$) but was marginally associated with OS ($P = 0.05$) with cases in the high category group having worse survival. In this series of breast tumors, CVC failed to achieve significance for either OS ($P = 0.1$) or RFS ($P = 0.1$). A multivariate analysis including size, node status, ER, EGFR, and TAM HIF-2α expression showed no significant association between TAM HIF-2α and either RFS or OS (Table 2).

Discussion

This study found that increasing numbers of HIF-2α-positive macrophage clusters within invasive breast carcinomas correlated closely with increased tumor angiogenesis, as well as worse survival. High HIF-2α TAM expression was a feature of high-grade tumors. These results extend our previous study, which found that increased macrophage index was associated with both increased angiogenesis and worsened survival (2); although the present study was performed on a largely new consecutive series of cases, there was a 30% overlap with that of the previous study.

Previous staining studies have shown high levels of HIF-1 expression in tumor cells of several different tumor types, such as clear cell renal cancer (11). HIF-1 has also been shown to be an indicator of poor prognosis in oropharyngeal cancer (12), where it was found to have prognostic significance in patients undergoing radiotherapy. Conversely, in lung tumors, high HIF-1 expression has been shown to correlate with good prognosis (13). We have shown previously that unlike HIF-1, HIF-2α staining is greater in TAMs than in the neoplastic cells (7) and that hypoxia will induce HIF-2α expression in the macrophage-like cell line U937. We have also shown that in breast tumors, macrophages migrate to areas distant from angiogenic hotspots (2), suggesting that tumor hypoxia is responsible for the induction of HIF-2α expression in these macrophages.

We have also shown that increased focal macrophage infiltration into tumors was associated with increased angiogenesis (2). This study now suggests that the association with angiogenesis may involve the up-regulation of HIF-2α specifically in these hypoxic macrophage clusters. It has long been established that macrophages are powerful stimulators of angiogenesis via secretion of a number of direct and angiogenic factors (1). The specific mechanism for the induction of angiogenesis by TAMs in breast tumors has yet to be established; however, it would seem from the evidence presented here that it may involve the activation of HIF-2-responsive genes in macrophages. These could potentially be genes for angiogenic cytokines or indirectly angiogenic peptides, such as proteolytic enzymes. Another intriguing possibility is that the macrophage-derived angiogenic peptide PR39 may be up-regulating HIF-2α. PR39 stimulates angiogenesis by inhibiting the degradation of HIF-1α by the ubiquitin-proteosome pathway (14). In breast cancer, this would lead to increased transcription of HIF-1-responsive genes, such as VEGF by the tumor cells, the consequence of which would be increased angiogenesis and increased macrophage infiltration (as VEGF is also a potent monotactic factor; Ref. 15). It would be an interesting future study to assess whether the *PR39* gene inhibits HIF-2 degradation.

Previous studies on cell lines demonstrate that epithelial tumor cell lines generally have higher levels of HIF-1α than HIF-2α (6). In

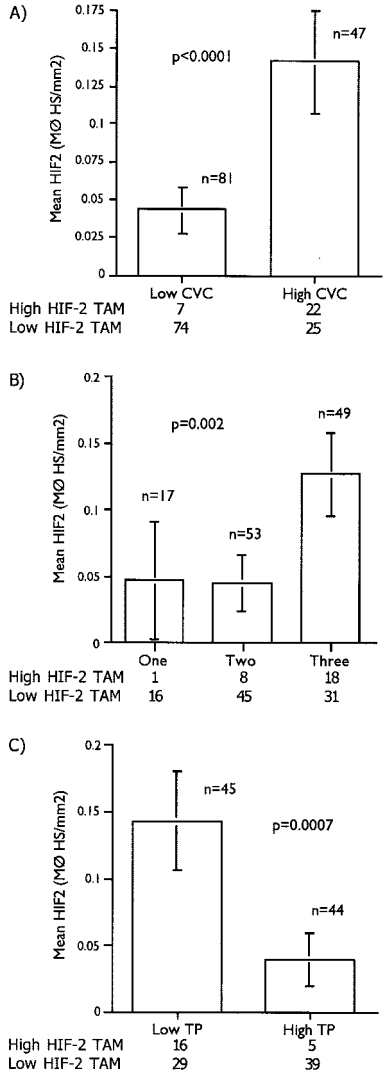

Fig. 2. *A*, graph showing association between high macrophage HIF-2α expression and increased angiogenesis in invasive breast tumors. *B*, graph illustrating the increased HIF-2α staining found in grade 3 ductal carcinomas. *C*, graph showing inverse relationship between expression of macrophage HIF-2α and tumor TP.

macrophage and endothelial cell lines (which share a common lineage), the opposite is true with the expression of HIF-2α being higher than HIF-1α (7). Thus, it would seem that these cells could have a different hypoxia program than epithelial-derived cells, with a different set of hypoxia-inducible genes, *e.g.*, the angiopoietin receptor tie-2 (16).

It was also notable that high HIF-2α TAM expression was a feature of high-grade breast tumors. Grade 3 tumors are highly aggressive and fast growing and often contain areas of necrosis within them. Because of tumor heterogeneity, it has not been possible to clearly demonstrate that high-grade tumors are more hypoxic than those of lower grade, but these findings suggest that high-grade tumors could have hypoxic areas within them with consequent induction of hypoxia-regulated genes. This would be consistent with the hypothesis that hypoxia is a major stimulus behind angiogenesis and macrophage infiltration with consequent up-regulation of HIF-2α. Indeed, Grimshaw *et al.* (17) have found that macrophages are trapped in hypoxic areas and that this inhibition of migration out of the hypoxic area is mediated by HIF-1 modulation of mitogen-activated protein kinase phosphatase-1 activity, which down-regulates the chemoattractant signaling cascade. Thus, it appears that both HIF-1 & 2 are involved, with perhaps HIF-1 controlling macrophage trafficking and HIF-2 controlling the angiogenic activation.

Another finding of this study was an inverse relationship between tumor TP expression and TAM HIF-2α. A hypoxia response element is not present in the *TP* gene; although it has been reported to be induced by hypoxia in one cell line (18), it has not been found to be hypoxically regulated in any other. TP is an angiogenic factor commonly expressed in breast tumors (10), and it can be induced by TAM-derived cytokines, such as tumor necrosis factor-α (8), which suggests two alternative TAM-mediated angiogenic pathways: (*a*) hypoxic tumors via induction of TAM HIF-2α; and (*b*) in the presence of oxygen, TP may induce carcinoma cell oxidative stress via the generation of reactive oxygen species, which up-regulate the potent angiogenesis-related factors VEGF, matrix metalloprotease-1, and interleukin-8 (9). These pathways seem to be reciprocal in that one or another dominates, perhaps reflecting tumor hypoxia or oxygenation selecting the relevant pathway for angiogenesis.

These findings, although not conclusive, would be consistent with the following sequence of events. As aggressive tumors outgrow their blood supply, hypoxia induces TAM clustering and overexpression of TAM HIF-2α. Hypoxia activates these TAMs to express an angiogenic phenotype, leading to the induction of localized angiogenic hot spots. This would account for the correlation between TAM HIF-2α and angiogenesis. Conversely, in less hypoxic tumors, which are still dependent on angiogenesis, alternative nonhypoxia-dependent TAM angiogenic pathways may be present.

With regards to prognosis, in a univariate analysis, HIF-2α TAM expression did achieve marginal significance for OS but not RFS. However, this association was not confirmed in a multivariate analysis ($P = 0.06$). We have shown previously that macrophage index is a powerful prognosticator in breast cancer, with high levels of focal macrophage infiltration correlating with worsened RFS and OS. HIF-2α-expressing macrophages are found much less frequently outside the tumor environment. Some HIF-2α expression has been observed in kuppfer cells and a subpopulation of bone marrow macrophages, and it has been suggested that this is a feature of some stages of differentiation or specific types of activation (7). Because of the strong association, possibly mechanistically, with other pathological variables, such as grade, it is not possible to see an independent effect, but it may be that this contributes to the well-recognized effects of grade on prognosis.

Recently, it was shown that blockading the interaction of HIF-1 with its transcriptional coactivators inhibited hypoxia-inducible gene expression and retarded tumor growth (19). Our results suggest that a similar approach targeting HIF-2 signaling in TAMs could also be considered as a possible new area of antiangiogenic tumor therapy. Similarly, there are inhibitors of TP that are antiangiogenic (20). The effectiveness of either alone may depend on the angiogenic profiles in the tumor; however, it is clear that combined approaches to block oxygen-dependent and -independent pathways may be necessary in the clinical situation, as well as careful evaluation of patients in trials with these drugs.

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