The Role of Hypoxia-Inducible Factor-1 in Three-dimensional Tumor Growth, Apoptosis, and Regulation by the Insulin-Signaling Pathway

Russell D. Leek, Ian Stratford, and Adrian L. Harris

Growth Factor Group, Cancer Research UK Molecular Oncology Laboratories, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford, United Kingdom and School of Pharmacy and Pharmaceutical Sciences, University of Manchester, Manchester, United Kingdom

Abstract

The purpose of this study was to establish the effect of hypoxia-inducible factor-1 (HIF-1) directly on tumor growth, independently of angiogenesis. This was done by growing wild-type mouse hepatoma cells (HEPA-1) and their HIF-1-deficient counterpart C4 as multicellular tumor spheroids and quantifying differences in growth rates and proliferative and apoptotic indices. Insulin and insulin-like growth factor-I are key growth factors, also able to regulate hypoxia-responsive genes via HIF-1; thus, the effects of insulin on this model were also investigated. Two-dimensional growth was serum dependent and no difference was seen between wild-type HEPA-1 and C4 cell growth profiles, but major differences were seen in three-dimensional growth. HIF-1 supported spheroid growth under hypoxia as the HEPA-1 spheroids grew faster than the C4. Surprisingly, the HIF-1–deficient cells had a higher proliferation rate in three-dimensional growth (C4 mean S-phase index, 13.6%; HEPA-1 mean S-phase index, 9%; \( P = 0.009 \)) that was associated with an inhibition of apoptosis. However, the apoptosis rate was much greater in these spheroids (C4 mean apoptotic index, 6.4%; HEPA-1 mean apoptotic index, 0.78%; \( P = 0.0006 \)). Addition of insulin increased proliferation and apoptosis in both HEPA-1 and C4 spheroids, demonstrating an HIF-1–independent effect of insulin signaling in three-dimensional growth. These results indicate that the enhancing effect of HIF-1 in three-dimensional tumor growth is a balance of both reduced proliferation and enhanced survival, the latter being proportionally greater. (Cancer Res 2005; 65(10): 4147-52)

Introduction

Hypoxia is common in human cancer (1) and stabilizes hypoxia-inducible transcription factor HIF-1α. The DNA binding complex of HIF-1 is a heterodimer composed of two helix-loop-helix proteins HIF-1α and HIF-1β (2). The HIF-1 protein complex mediates transcriptional responses to hypoxia by binding to hypoxia response elements on specific target genes such as VEGF (2). The role of HIF-1α in tumor growth and development is still uncertain, as a number of studies have produced varying results. Thus, loss of HIF-1α in embryonic stem–derived tumors (3). However, disruption of the HIF-1α locus resulted in accelerated growth of teratocarcinomas (4). Some studies showed that loss of HIF-1 function inhibits both angiogenesis and tumor growth (5–7); others show impaired growth but no effect on angiogenesis (3, 6, 8, 9). Blouw et al. have shown that astrocytomas deficient in HIF-1α grow faster in vascular areas of the brain than wild-type tumors, but the same tumors grown in a vessel poor s.c. environment has slower growth, indicating that HIF-1 may have a differential role in tumor progression depending on the microenvironment (10). These data show the conflicting effects of HIF-1, we therefore analyzed some of the components in an in vitro model of three-dimensional growth to understand the role of HIF-1 in growth, independently of the effects of angiogenesis, which clearly could mask or modify the actions of HIF-1 in vivo. Cell lines chosen for this study were the mouse hepatocellular carcinoma cell line HEPA-1, the C4 variant of which has a mutation that renders it deficient in HIF-1 (11). In the C4 variant, HIF–1-responsive gene induction under hypoxia is impaired, and we previously reported that xenografts of the C4 cell line grow more slowly and were markedly less vascularized than their HEPA-1 wild-type (WT) counterparts (5). By growing the C4 mutant and the wild-type HEPA-1 cell lines as multicellular spheroids, it was possible to assess whether HIF-1α was a requirement for tumor growth independent of angiogenesis. Insulin and insulin-like growth factor-I (IGF-I) are key growth factors and can regulate expression of hypoxia responsive genes, by the induction of HIF-1α (12–14). HEPA cells are known to express the insulin receptor but not the IGF-1 receptor (12) so this provides a cell type to investigate whether effects of insulin on growth are mediated via HIF. The purpose of this study was, therefore, to establish whether HIF-1 was involved in tumor growth by effecting either proliferation or apoptosis, and to examine the effects of insulin on these pathways.

Materials and Methods

Spheroid culture and measurement. HEPA-1 and C4 cells were cultured to 95% confluence and seeded into agarose-coated 96-well plates at a density of 2,000 cells per well and cultured. Each well contained 200 μL of tissue culture medium, and the spheroids were fed every other day by carefully aspirating 100 μL of spent medium and replacing with the same quantity of fresh medium. To calculate mean sizes, diameters of 20 spheroids were measured every 1 to 3 days. Each experiment was repeated thrice and presence of necrosis was noted.

Additionally, 96-well plates of spheroids were harvested and fixed in formalin. After processing into wax blocks, the spheroids were sectioned and stained with H&E. Using a microscope eyepiece, micrometer-scale overall diameter, diameter of necrotic zone, and viable rim thickness were measured.

Bromodeoxyuridine, caspase-3, and p27 labeling. Spheroids were incubated in 10 μmol/L bromodeoxyuridine (BrdUrd; Calbiochem, La Jolla,
CA) for 30 minutes before harvesting and fixation. Sections were stained using a BrdUrd streptavidin/biotin peroxidase staining kit (Oncogene Research Products, Cambridge, MA). Total nuclei numbers and numbers positive for BrdUrd per section were counted, yielding a mean proliferative index (% positive nuclei).

To assess apoptosis, spheroids were immunohistochemically labeled for caspase-3. A rabbit polyclonal antibody to mouse caspase-3 (R&D Systems Europe Ltd., Abingdon, United Kingdom) was used at a concentration of 0.1 µg/mL for 30 minutes. A mean apoptotic index was calculated for proliferative index.

**Figure 1.** A, growth curves of HEPA-1 and C4 cell lines in monolayer culture. B, growth curves of HEPA-1 and C4 cell lines in three-dimensional multicell spheroid culture. Bars, SE (too small to be visible). *, P < 0.0001 (see Table 1A). C, series of images of H&E-stained HEPA-1 and C4 spheroids showing actual size differences with increasing age. D-G, effects of insulin, serum-free medium, and PI 3-kinase inhibitor on growth curves of HEPA-1 and C4 cell lines in spheroid culture. Bars, SE (too small to be visible). Comparisons of different curves: #, P = 0.02; †, P = 0.01; +, P = 0.004; Ø, P = 0.031; **, P = 0.019; ‡, P = 0.05; ††, P = 0.027 (summarized in Table 1B-G).
P27 was labeled on sections using microwave antigen retrieval in 0.01 mol/L citrate buffer (pH 6) for 4 + 4 minutes, a mouse monoclonal antibody to mouse p27 (SC-1641, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used at a concentration of 2 μg/mL for 30 minutes. A mean p27 index was calculated for proliferative index.

**Measurement of cell density in monolayer culture.** Monolayer cultures were initially seeded in a 75-cm² flask and the growth rate analyzed by counting cells in a hemocytometer.

**Treatment of HEPA-1 and C4 cell lines with insulin and LY294002 in monolayer and spheroid culture.** After 3 days, subsets of HEPA-1 and C4 spheroids and monolayers were cultured with either normal medium containing 10% FCS or serum-free medium, or serum-free medium containing insulin at concentrations of 25, 100 nmol/L, or 1 μmol/L. Additionally, some spheroids were also cultured in medium without serum and with 100 nmol/L insulin, plus the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor LY294002 (Sigma Laboratories, St. Louis, MO) at a concentration of 20 μmol/L.

**Statistical analysis.** Unpaired and paired t tests were used when comparing growth rates, S-phase labeling index, and apoptosis. Results for all time points were pooled and compared between cell lines or experimental condition. P values < 0.05 were regarded significant.

**Results**

**HEPA-1 and C4 monolayers growth curve analysis.** HEPA-1 and C4 monolayers grew at similar rates over 7 days (Fig. 1A). Neither grew in the absence of serum but with 100 nmol/L insulin, both grew more quickly (Fig. 1D). In hypoxic conditions neither cell line grew (data not shown).

Additionally, some spheroids were also cultured in medium without serum and with 100 nmol/L insulin, plus the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor LY294002 (Sigma Laboratories, St. Louis, MO) at a concentration of 20 μmol/L.

**Table 1. Statistical comparisons of HEPA-1 and C4 spheroids**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Figure</th>
<th>Cell line</th>
<th>Condition</th>
<th>Mean diameter (μm)</th>
<th>Volume (×10⁶ μm³)</th>
<th>P</th>
<th>Volume change</th>
</tr>
</thead>
<tbody>
<tr>
<td>A *</td>
<td>1B</td>
<td>C4</td>
<td>After 6 d</td>
<td>439 (3.3)</td>
<td>44.18</td>
<td>&lt;0.0001</td>
<td>2.26-fold increase</td>
</tr>
<tr>
<td>B #</td>
<td>1D</td>
<td>HEPA-1</td>
<td>After 6 d</td>
<td>516 (43.1)</td>
<td>71.76</td>
<td>0.02</td>
<td>2-fold increase</td>
</tr>
<tr>
<td>C §§</td>
<td>1E</td>
<td>C4</td>
<td>After 6 d</td>
<td>421 (4.1)</td>
<td>45.1</td>
<td>0.01</td>
<td>2.7-fold increase</td>
</tr>
<tr>
<td>D omega</td>
<td>1D</td>
<td>HEPA-1</td>
<td>Without inhibitor</td>
<td>516 (43.1)</td>
<td>71.76</td>
<td>0.031</td>
<td>83% reduction</td>
</tr>
<tr>
<td>E **</td>
<td>1E</td>
<td>C4</td>
<td>Without inhibitor</td>
<td>424 (24.1)</td>
<td>45.1</td>
<td>0.019</td>
<td>76% reduction</td>
</tr>
<tr>
<td>F §§</td>
<td>1F</td>
<td>HEPA-1</td>
<td>With insulin + LY294002</td>
<td>443 (19.6)</td>
<td>45.41</td>
<td>0.05</td>
<td>67% reduction</td>
</tr>
<tr>
<td>G #</td>
<td>1G</td>
<td>C4</td>
<td>With insulin</td>
<td>616 (41.5)</td>
<td>122.1</td>
<td>0.027</td>
<td>77% reduction</td>
</tr>
<tr>
<td>G #</td>
<td>1G</td>
<td>C4</td>
<td>With insulin + LY294002</td>
<td>377 (13.1)</td>
<td>27.99</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**HEPA-1 and C4 spheroid growth curve analysis.** H&E-stained sections of HEPA-1 WT and C4 spheroids were measured morphometrically and showed a difference in growth profile (Fig. 1B and C). Growth rates diverged beginning at 72 hours (3 days) when the spheroids reached a diameter of 200 μm (equivalent to a volume of 4.18 × 10⁶ μm³) just as central necrosis appeared (Fig. 1B). After 168 hours (7 days), the difference was marked and in vitro growth of the C4 mutant spheroids was retarded compared with the wild-type HEPA-1 spheroids (Fig. 1B and C; Table 1A).

Necrosis appeared in both spheroids at a diameter of 250 μm (equivalent volume of 8.1 × 10⁶ μm³), occurring sooner in the HEPA-1 WT [120 hours (5 days) compared with 168 hours (7 days) in the C4s]. After 360 hours (15 days), the growth rates leveled off in both cell lines, and both types of spheroid sloughed off cells into the surrounding media. In contrast to two-dimensional growth, the growth rate of both types of spheroid was not dependent upon the presence of FCS (Fig. 1D and E).

**Effect of insulin on HEPA-1 and C4 spheroid growth, and effect of PI 3-kinase inhibitor.** Growth rates were enhanced in HEPA-1 when grown in the presence of insulin (Fig. 1D and F; Table 1B), as were C4 spheroids (Fig. 1E and G; Table 1C). Insulin stimulated growth of the C4 cell line to a rate higher than that of the HEPA-1 spheroids grown in medium containing 10% FCS (Fig. 1D and G; P = 0.004).

To determine whether insulin-enhanced growth was mediated partly via a PI 3-kinase-dependent pathway, the effect of the...
PI 3-kinase inhibitor LY294002 was analyzed. LY294002 dramatically retarded growth of both types of spheroid when cultured with and without 100 nmol/L insulin (Fig. 1D-G; Table 1D and E).

In LY294002-treated HEPA-1 and C4 spheroids, growth was immediately halted and the spheroids shrank (Fig. 1D). In LY294002-treated spheroids grown in 100 nmol/L insulin, growth was initially retarded until day 5, after which the spheroids shrank (Fig. 1F and G; Table 1F and G).

S phase, apoptosis, and P27 indices of HEPA-1 and C4 spheroids. In HEPA-1 spheroids, a pattern of S-phase labeling in the outer rim of four to five cells depth was observed. The density of labeling diminished towards the zone of necrosis. Surprisingly, the C4s showed an increase in labeling in early growth that was distributed more evenly throughout the spheroid (Fig. 2A). C4 spheroids had higher S-phase labeling indices than HEPA-1 during the initial 200 hours growth phase (Fig. 3A and B; Table 1H). Once the spheroids reached a size where the center became necrotic and the overall growth rate began to slow down (around 450-μm diameter), proliferation began to tail off in the C4 and HEPA-1 spheroids (Fig. 3A and B). Proliferation rates did not recover in the C4 spheroids but did in HEPA-1 spheroids after 200 hours.

Insulin increased proliferation in both spheroid types. Proliferation was higher in the C4 spheroids until they reached a diameter of 450 μm (Fig. 3C and D; Table 1I), when it dropped in both types with recovery only in the HEPA-1 spheroids.

The C4s had more apoptosis than HEPA-1 spheroids in the viable rim and near the necrotic core. The caspase-3 labeling was distributed throughout the viable rim in the C4 spheroids in contrast to the HEPA-1 spheroids where most labeling was confined to the perinecrotic area (Fig. 2B). Overall, apoptotic indices were significantly higher in C4 spheroids than HEPA-1 when untreated with insulin (Fig. 3A and B; Table 1J).

In HEPA-1 spheroids treated with insulin, apoptosis increased after 120 hours compared with untreated HEPA-1 spheroids (Fig. 3A and C; Table 1K).

Insulin did not increase apoptosis significantly in the C4 spheroids (P = 0.7), which was already much higher than in the HEPA cells (Fig. 3B and D). In both cell lines, insulin stimulated the appearance of apoptosis earlier than in the controls.

p27 expression was significantly higher in HEPA-1 spheroids than C4 spheroids at all time points between 3 and 15 days (Figs. 2C and 3E; Table 1L).

Discussion

Although in normal monolayer culture, the growth rates of the HEPA-1 and C4 cells were very similar, in three-dimensional spheroid culture, a marked difference between growth rates was seen, with the HEPA-1 cells having a distinct growth advantage over the ARNT mutant C4 variant from the third day of culture onwards, as hypoxia developed.

In a three-dimensional spheroid culture, there are gradients for glucose, lactate, pH, and oxygen which are far greater than obtained in two-dimensional growth where there is a larger surface area to volume, the depth of the fluid medium is the major limiting factor for oxygen diffusion, and all cells have equal contact with the medium. The latter is in striking contrast to spheroids where only the outer layer of cells has direct contact with the medium which simulates the realistic situation in vivo where many cells are ≥100 μm away, often 2 to 300 μm away, from a blood vessel. Thus, the spheroids generate a much stronger signal for the relevant pathways, which are never recapitulated in two-dimensional growth and enhance the elucidation of HIF function.

Tumor growth is a function of cell gain by division over cell loss by apoptosis. Considering the potential tumor promoting role of HIF-1, we were surprised to find in this study that S-phase indices in the HIF-1–deficient C4 cells where higher than in the normal HEPA-1 WT cells. However, recently Wang et al. (15) have shown that hypoxia causes an HIF-1α–dependent increase in the cyclin-dependent kinase inhibitor (CDKi) p27 and also showed that loss of HIF-1α causes an increased progression into S phase and abolishes hypoxia induced growth arrest. Meanwhile, Goda et al. observed that HIF-α is required for cell cycle arrest during hypoxia and that BrdUrd labeling was increased in HIF-α null B cells in culture (16), an observation that has also been shown in HIF-α null chondrocytes in vivo (17). Taken together, these findings would seem contradictory to our observation that the overall growth rate was slower in the C4 spheroids. Other studies have shown that...
HIF-1–defective tumor cell lines can grow more quickly than those with functional HIF-1 in normoxia (6), but we observed no difference in the growth rates of the WT and HIF-1 dysfunctional cell lines in normoxic monolayer culture.

However, when the apoptotic indices were compared, the C4 spheroids had much greater fold levels of apoptosis than the wild-type HEPA-1 cells (5.6% of cells in the C4 spheroids compared with 0.6% in the HEPA-1), and expression levels of p27 were higher in the HEPA-1 spheroids than those of the C4 cell line, explaining why proliferation rates were generally lower in the HEPA-1 spheroids. Thus, in the spheroids HIF-1 has a dual role in the regulation of cell division and resistance to apoptosis. Hypoxia causes cell death partly involving the proapoptotic HIF-regulated factor BNip3 (18–20). Indeed, the necrotic center developed earlier in the HIF-1 functional cells. Nevertheless, in the spheroid model, overall HIF-1 has an antiapoptotic effect as measured by inhibiting caspase-3 activation in the proliferating compartment and final growth rate of the spheroid.

It is likely that this represents two different mechanisms: at the higher oxygen tensions that also allow proliferation, HIF-1 would protect from apoptosis by restricting proliferation in suboptimal conditions to meet available nutritional resources. In contrast, in this modest hypoxia, the C4 spheroids have apoptosis scattered throughout the viable rim, whereas it only occurs near the necrotic zone in wild-type cells. However, both cell types have a necrotic core implying that there may be other cell death pathways that are HIF-1 independent ultimately causing death under severe hypoxia. We recently showed the ATF4 is such a pathway activated by protein stabilization under anoxia and independent of HIF-1 (21). This model provides a way to analyze the non-HIF-1 hypoxic cell death routes. Mechanisms to bypass these may be important in tumor evolution.

Insulin has been reported to signal via the HIF-1 pathway and activation of PI 3-kinase has a role in survival under hypoxia. In this study, insulin increased the growth rate of both cell lines in monolayer culture by a similar amount. In spheroid culture, insulin

![Figure 3](image_url)

**Figure 3.** A–D, S-phase and apoptotic indices of HEPA-1 and C4 cells grown with and without insulin in three-dimensional spheroid culture. Bars, SE (too small to be visible). Comparisons of different curves: *, P = 0.009; ø, P = 0.007; #, P = 0.0006; †, P = 0.0002 (summarized in Table 1 H-K). E, P27 labeling indices of untreated HEPA-1 and C4 spheroids. ***, P = 0.03 (see Table 1L).
also increased the growth rate of both cell lines, showing HIF-1 was not necessary for its effects. Thus, the growth signaling response to insulin, in contrast to other works (22), is via an HIF-1–independent mechanism. To investigate this further, the PI 3-kinase inhibitor LY294002 was used to blockade the PI 3-kinase pathway. The result was growth retardation in both cell lines when grown as spheroids without insulin. The addition of insulin with the inhibitor then lead to some recovery in both cell lines. This implies that insulin growth signals are mediated mainly through an HIF-1–independent PI 3-kinase–dependent pathway. These results may reflect the analysis of signaling in three-dimensional growth, which is likely to be more relevant to the in vivo pathways (16, 18–20).

Because of the possibility of serial analysis in the spheroids, in contrast to previous in vivo tumor studies of the role of HIF-1, we were able to observe the time course of proliferation and apoptosis after stimulation by insulin. In the wild-type HEPA-1 spheroids, increased proliferation was accompanied by increased apoptosis that occurred at the same time as enhanced growth, perhaps because of forced proliferation in unfavorable physiologic circumstances. Proliferation did recover in the HEPA-1 cells after a period of growth slowing. Interestingly, no further increase in apoptosis could be induced in the mutant cells. This result again shows the complexity of the relation of HIF-1 function to hypoxic death. Moderate degrees of hypoxia induced protection from apoptosis via HIF-1. The more severe stresses inducing apoptosis via a pathway that can bypass HIF-1. It is likely there will be a maximum apoptotic response that can be achieved by any particular pathway and in the mutant cells, that was already reached by loss of HIF-1 protection. In wild-type cells, it is only induced under more extreme circumstances with insulin-stimulated proliferation. Hence, there are similar maximum levels in both cell types.

These results show that HIF-1 is a dominant factor regulating tumor growth and helps explain the conflicting in vivo results reported as angiogenesis could compensate, depending on its extent, for hypoxia and the overall effect will be determined by relative balance of proliferative and apoptotic pathways in the tumor. Apoptosis was higher in the C4 cells, which accounts for the higher overall growth rate of the HEPA-1 spheroids and also supports the reasoning that HIF-1 is a major survival factor suppressing apoptosis. In our study, enhanced production of a specific growth factor could compensate for a defect in HIF-1 and this is relevant to a potential resistance mechanism to therapy that inhibits HIF-1. The importance of the vascular interaction in confounding the role of HIF-1 is shown in the work of Blouw et al. (10), where HIF-1α knock-out inhibited growth s.c., with inhibition of angiogenesis, but was associated with enhanced proliferation in the brain, where vascular option was possible.

Overall, our results support the concept that HIF-1 could be used as a target for antitumor therapy because it is an important factor in three-dimensional tumor growth. In this system, HIF-1 suppresses proliferation and prevents against apoptosis in hypoxic conditions that would interfere with traditional chemotherapy regimes, so inhibition would produce a high proliferation index and enhance apoptosis and therefore likely to synergize with cytotoxic agents.

Acknowledgments


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