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Defining hypoxic microenvironments by non-invasive functional optical imaging

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Abstract Functional imaging has become an important tool in oncology by informing about localisation and size of the tumour as well as the pathophysiological features of tumoural cells. One of the most characteristic features of some tumour types is the activation of the neoangiogenic programme which is specifically mediated by the transcription factor hypoxia-inducible factor (HIF)-1α, an important player in regulating this process and a prognostic marker of tumoural aggressiveness. Here we report a non-invasive *in vivo* detection of lung micrometastases in a mouse model of breast cancer using self-illuminating genetically encoded tracers responsive to intracellular HIF-1α levels and a preliminary analysis of the contribution of the tumoural masses to the metastatic niche. This model lays the foundations for novel hypoxia sensing probes able to detect micrometastatic disease with high sensitivity and specificity. Thus, optical functional imaging shows promise in the understanding of disease, drug development or image-guided therapy.

1. Introduction

The monitoring of biological processes in a functional non-invasive way has been a major milestone in basic and clinical research in recent years, and especially in oncology. Functional imaging gives more detailed information about tumoural physiological processes such as oxygenation rate, perfusion and alterations of blood flow. As an example, a variation of the magnetic resonance imaging (MRI) technique, functional MRI (*f*MRI) is currently used to map areas of the cerebral cortex in relation to brain cancers.[1] Optical imaging also allows the possibility of shifting from structural to functional imaging by revealing pathophysiological features within certain tissue, because it is able to translate specific molecular and cellular processes associated to a disease to changes in light emission. Besides, these methods do not display the harming effects of ionising radiations on living organisms or require high-budget equipment to monitor the overall process as MRI does.[2] However, although fluorescence imaging (FLI) is known to face

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problems when it comes to autofluorescence in whole-body imaging this can be circumvented by using probes able to emit light in the near infrared part of the spectrum or even higher. And while bioluminescence imaging (BLI) suffers from signal scattering and poor spatial accuracy, new advances in reconstructive tomography intend to overcome these technical difficulties enhancing BLI spatial resolution and quantification.[3,4]

In cancer biology, hypoxia/hypoxia-inducible factor (HIF)-1 pathway seems to play a key role in invasion and metastasis. HIF-1 is a central regulator of hypoxic gene expression and involved in the restoration of cellular oxygen homoeostasis. HIF-1 is a HIF-1α/HIF-1β heterodimer that binds the hypoxia response elements (HREs) of target genes under hypoxic conditions. While HIF-1β is constitutively expressed, the expression and transcriptional activity of HIF-1α is precisely regulated by intra-cellular O_2 concentrations.[5] Under normoxia, prolyl hydroxylases modify Pro-402 and Pro-564 of HIF-1α in a reaction that uses O_2 as a substrate. Then, hydroxylated HIF-1α interacts with von Hippel–Lindau (VHL), which is part of an E3 ubiquitin ligase complex targeting HIF-1α for 26S proteasomal degradation. On the other hand, under hypoxic conditions, HIF-1α is stabilised because of the lack of O_2 and dimerises with HIF-1β to bind to the HREs. In a coordinated fashion with coactivator CBP/p300, HIF-1 activates the transcription of target genes involved in such diverse programmes as glucose transportation and glycolysis, angiogenesis, survival and proliferation, or invasion and metastasis. Mutations in tumour suppressor genes, such as *PTEN* and *VHL*, hyperactivation of oncogenic pathways (i.e. Ras/mitogen-activated protein kinase (MAPK) and PI3K/AKT pathways) or the presence of reactive oxygen species (ROS) can also regulate HIF-1α in an oxygen-independent manner. Accordingly, HIF-1α overexpression is usually linked with poor prognosis in several cancer types since it correlates with high metastatic potential of these tumoural cells.[6,7]

Another kind of transcription factors, the signal transducer and activator of transcription (STAT) protein family, is also regulated by hypoxia and oxidative stress, besides other cytokines and growth factors. These transcription factors exist in an unphosphorylated latent form in the cytoplasm until undergoing phosphorylation by a number of tyrosine kinases. When activated, these proteins play a dual role by acting as signal transducers in the cytoplasm and transcriptional activators in the nucleus.[8] Aberrant activation of epidermal growth factor receptor kinases, especially HER1/erbB-1 and HER2/neu, and overexpression of EGF ligands are linked with breast carcinoma progression,[9,10] and result in downstream hyper-activation of STAT proteins. Although STAT proteins are upregulated in tumoural cells, only STAT3 and STAT5 are thought to have a relevant role in oncogenic development.[11] In keeping with this, constitutive STAT3 activation occurs frequently in a variety of human tumour cell lines, namely breast cancer,[12,13] leukaemia,[14] prostate cancer,[15] melanoma[16] and myeloma.[17] It also reveals the fact that this member of the JAK-STAT pathway maintains a crosstalk with the hypoxia response, as several reports suggest that STAT3 is required conjointly with HIF-1α for maximum induction of vascular endothelial growth factor (VEGF) under hypoxic conditions or via Src.[18]

2. Methods

2.1. Establishment and characterisation of cell sublines

Human breast cancer cells MDA-MB 231 were cultured in Dulbecco's modified Eagle medium, DMEM (Sigma–Aldrich, MO, USA), supplemented with 10% foetal bovine serum (Fisher Scientific, PA, USA). Cell cultures were maintained at 37 °C and 5% CO_2. DNA transfections were carried out using the Effectene transfection reagent (Qiagen, CA, USA). Cells were seeded in 12-well plates at a cellular density of 5×10^4 cells/plate. Each well was transfected with 10:1 or 5:1 M ratios of E-M-H-mCherry-Luc and pMC1neo-polyA (Stratagene, CA, USA). Cell cultures were maintained under antibiotic selection at a concentration of 1 μg/mL of G418 (Sigma–Aldrich, MO, USA) for 2 weeks. Cells from each subline (ECL-A5, ECL-A10, ECL-B5, ECL-B10) were seeded at a density of 1×10^6 cells/plate and in order to induce an artificial hypoxic environment each pool was treated with $CoCl_2$ (Sigma–Aldrich, MO, USA) at a concentration of 500 μM. Also, L-Mimosine (Sigma–Aldrich, MO, USA), another hypoxia-mimetic agent, was added to each pool up to a final concentration of 800 μM. All cells were trypsinised 24 h later, collected by centrifugation, washed thoroughly with PBS to eliminate traces of medium and resuspended in 1× PBS. Fluorescence acquisition was performed as previously reported,[19] using an IVIS Spectrum (Caliper LS, CA, USA). Cells from ECL-B10 subline were serially diluted in 96-well plates and D-Luciferin was subsequently added to each plate at a final concentration of 150 μg/mL before imaging. Imaging time was 1 min/plate. All experiments were carried out three times at least.

2.2. Cell implantation and fluorescence-bioluminescence in vivo assays

Cells from ECL-B10 cell line were seeded and trypsinised 48 h later with Trypsin (Fisher Scientific, PA, USA), collected by centrifugation and resuspended in sterile 1× PBS at a concentration of 2×10^3 cells/μL. Seven BALB/c nude mice were injected intravenously (i.v.) each with 2×10^5 cells (100 μL). Upon sedation with isoflurane (2%) using the XGI-8 Gas Anaesthesia Unit fluorescence and bioluminescence data were registered with an IVIS Spectrum®. Fluorescence images