Nitric Oxide Is a Factor in the Stabilization of Hypoxia-Inducible Factor-1α in Cancer: Role of Free Radical Formation

Marisol Quintero,1 Peter A. Brennan,3 Gareth J. Thomas,2 and Salvador Moncada1

1The Wolfson Institute for Biomedical Research, University College London;2Tumour Biology Laboratory, Cancer Research UK Clinical Centre, Queen Mary’s Medical and Dental School at Bart’s and the London, London, United Kingdom; and 3Department of Oral and Maxillofacial Surgery, Queen Alexandra Hospital, Portsmouth, United Kingdom

Abstract

Widespread expression of the α-subunit of hypoxia-inducible factor (HIF-1α) was observed in samples of human oral squamous cell carcinoma. In all the cases, this was accompanied by a widespread distribution of nitric oxide (NO) synthases (NOS). Furthermore, in three human cell lines derived from human oral squamous cell carcinoma, the accumulation of HIF-1α was prevented either by inhibition of NOS activity with the nonspecific NOS inhibitor L-NAME or by the antioxidants hydroxylases (PHDs; ref. 2). These enzymes, at physiologic O2 concentrations, constantly modify HIF-1α and target it for proteosomal degradation (3, 4). Following inhibition of PHDs, HIF-1α dimerizes with the constitutive HIF-1β to form HIF-1, which is responsible for activation of a variety of genes, including those involved in glycolysis, erythropoiesis, angiogenesis, and vascular remodeling (5). Although inhibition of PHDs due to hypoxia is recognized as the main mechanism responsible for the stabilization of HIF-1α, it has become evident that this can also occur by other mechanisms. Some growth factors and cytokines increase the synthesis of HIF-1α (see ref. 6), whereas some compounds that chelate iron seem to stabilize it, probably by acting on the protein itself or by affecting the action of the PHDs (7).

The presence of HIF-1α has been observed in different types of cancer and there is some indication that this is associated with malignancy and lack of response to treatment (8). Originally, the presence of HIF-1α in cancer was attributed to tumor hypoxia (9) but recent evidence suggests that other mechanisms may also be involved (10, 11). Increases in nitric oxide (NO) generation have been observed in certain forms of cancer, NO might be responsible for the accumulation of HIF-1α by a mechanism dependent on free radicals. (Cancer Res 2006; 66(2): 770-4)

Introduction

The dimeric transcription factor hypoxia-inducible factor (HIF-1) is a key regulator of the cellular response to hypoxia (1). Its nuclear translocation follows cytosolic accumulation of its α-subunit (HIF-1α), which results from inhibition of the O2-sensitive prolyl hydroxylases (PHDs; ref. 2). These enzymes, at physiologic O2 concentrations, constantly modify HIF-1α and target it for proteosomal degradation (3, 4). Following inhibition of PHDs, HIF-1α dimerizes with the constitutive HIF-1β to form HIF-1, which is responsible for activation of a variety of genes, including those involved in glycolysis, erythropoiesis, angiogenesis, and vascular remodeling (5). Although inhibition of PHDs due to hypoxia is recognized as the main mechanism responsible for the stabilization of HIF-1α, it has become evident that this can also occur by other mechanisms. Some growth factors and cytokines increase the synthesis of HIF-1α (see ref. 6), whereas some compounds that chelate iron seem to stabilize it, probably by acting on the protein itself or by affecting the action of the PHDs (7).

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Materials and Methods

Immunohistochemistry. Immunohistochemistry for inducible NOS (iNOS), endothelial NOS (eNOS), and HIF-1α proteins was carried out on 29 formalin-fixed specimens of human oral squamous cell carcinoma and 10 samples of normal oral mucosa. Monoclonal antibodies to iNOS, eNOS (BD Biosciences, Oxford, United Kingdom), and HIF-1α (Novus Biologicals, Littelton, CO) were used as previously described (16, 17), and samples were developed using an avidin biotin horseradish peroxidase system (DAKO, Glostrup, Denmark). Positive controls were normal kidney for HIF-1α and an oral squamous cell carcinoma known to have high eNOS expression and activity. Negative controls consisted of omitting the primary antibody to test for nonspecific secondary antibody binding and the use of irrelevant primary antibodies CD34 and p53, with the same isotypes as the primary antibodies being evaluated in this study, to assess for nonspecific primary antibody. Tumors were graded as showing no expression (negative) or expression (positive) for staining relative to known controls by two researchers using a conference microscope (Axioskop 2, Zeiss, Jena, Germany). Tumors were graded as HIF-1α positive only if both nuclear and cytoplasmic staining was seen (17).

Cells and reagents. Human umbilical venous endothelial cells (HUVEC) were purchased from PromoCell (Heidelberg, Germany). Cells were grown as previously described (18).

A panel of 11 oral squamous cell carcinoma cell lines was initially screened for NOS expression, and three cell lines (H157, H357, and BICR6, generously provided by Prof. S.S. Prime, University of Bristol Dental School, Bristol, United Kingdom and Prof. E.K. Parkinson, Institute of Dentistry, Queen Mary’s Medical and Dental School, Bart’s and the London, United Kingdom) were used for subsequent work.

Cells were grown in standard keratinocyte growth medium (KGM) as described (19). KGM comprised α-MEM containing 10% FCS (Globepharm, Surrey, United Kingdom) supplemented with 100 IU/L penicillin, 100 μg/L streptomycin, 1.8 × 10⁻⁴ mol/L adrenaline, 5 μg/mL insulin, 1 × 10⁻¹⁰ mol/L cholera toxin, 0.5 μg/mL hydrocortisone, and 10 ng/mL epidermal growth factor (Sigma, Dorset, United Kingdom). All cells were tested routinely for Mycoplasma.

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Requests for reprints: Salvador Moncada, The Wolfson Institute for Biomedical Research, University College London, The Cruciform Building, London WC1E 6BT, United Kingdom. Phone: 44 2076796666; E-mail: s.moncada@ucl.ac.uk.

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HIF-1 of the manufacturer to generate siRNA knock down HIF-1 RNA Technologies (Chicago, IL) and were used according to the instructions following genes:

Medhurst et al. (20) using SYBRGreen as a detection system for the Semiquantitative real-time PCR was carried out essentially as described by

Custom SMARTpool small interfering RNA (siRNA) reagents targeting HIF-1α or random (nontargeting siRNA) were purchased from Dharmacon (magnification, ×60). A, eNOS expression in blood vessels of normal mucosa (magnification, ×70). C, widespread presence of nuclear and cytoplasmic HIF-1α (magnification, ×60). *, prominent blood vessels.

Table 1. The immunohistochemical distribution of HIF-1α, eNOS, and iNOS

<table>
<thead>
<tr>
<th>Cases (n = 29)</th>
<th>HIF-1α</th>
<th>eNOS</th>
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<tr>
<td>14</td>
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<td>2</td>
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<td>–</td>
</tr>
<tr>
<td>Control (n = 10)</td>
<td>–</td>
<td>+*</td>
<td>–</td>
</tr>
</tbody>
</table>

NOTE: The expression of HIF-1α, eNOS, and iNOS was analyzed in 29 cases of oral squamous cell carcinoma and 10 samples of normal oral mucosa.

*Localized in blood vessels.
blot using a mouse monoclonal antibody (BD Biosciences) against HIF-1α, followed by an antimouse horseradish peroxidase conjugate (DAKO). The protein band was detected by enhanced chemiluminescence (Amersham Pharmacia). Activation of HIF-1 was quantified in 5 to 10 μg of nuclear extract by specific binding of HIF-1 to an oligonucleotide containing the HRRE for the Epo gene by means of the TransAM HIF-1 kit (Active Motif, Rixensart, Belgium) according to the instructions of the manufacturer.

Results

Immunohistochemical expression of HIF-1α and NOS in oral squamous cell carcinoma. A total of 29 carcinoma samples and 10 normal oral mucosal epithelial biopsies were analyzed for this study. In normal oral mucosa, eNOS was localized to blood vessel endothelium in the submucosa in all cases (Fig. 1A); iNOS and HIF-1α were not observed either in the epithelium or submucosa in any of the normal samples. The presence of eNOS, HIF-1α, and iNOS was detected in 25 of 29, 24 of 29, and 16 of 29 cancer cases, respectively (see Table 1). All three proteins were found in 14 of 29 cases, with eNOS and HIF-1α both being expressed in a further 10 of 29 cases. The expression of both HIF-1α and eNOS was observed diffusely throughout the entire tumor tissue, and there was no obvious correlation between either eNOS or HIF-1α and the distance from blood vessels (Fig. 1B and C, respectively). In the carcinoma samples, eNOS staining was seen in tumor cells and in the endothelium of both normal and tumor blood vessels (Fig. 1B). HIF-1α staining was also found to be both nuclear and cytoplasmic and was mainly localized to tumor cells (Fig. 1C). In no case was HIF-1α present in the absence of eNOS. The presence of iNOS was observed in 16 of 29 cases, being mainly localized to tumor cell cytoplasm, although some staining was also seen in peri-tumoral macrophages (not shown).

NOS in cancer cells. The presence of eNOS, iNOS, and nNOS was verified by Western blot. All three NOS isoforms were present in the cancer cell lines H357, H157, and BICR6; this was unlike the HUVEC in which only eNOS was found (Fig. 2). L-NMMA treatment significantly inhibited the generation of NO in BICR6 cells.
cells from 0.3 ± 0.05 (control) to 0.06 ± 0.02 nmol nitrite/5 × 10⁶ cells (treated).

**NO-dependent stabilization of HIF-1α in cancer cells.** The effect of endogenous NO on the stabilization of HIF-1α by lowering the O₂ concentration was analyzed in the three different cancer cell lines. Figure 3A shows that in BICR6 cells stabilization of HIF-1α occurs at high O₂ concentrations, so that it could even be observed at 21% O₂. As the O₂ concentration decreased, the stabilization of HIF-1α became more apparent. Figure 3B shows increases in nuclear binding of HIF-1α in BICR6 cells at different O₂ concentrations. The stabilization and binding were both reduced in the presence of L-NMMA. The NO donor DETA-NO restored the stabilization of HIF-1α in cells that had been treated with L-NMMA, confirming that the effect of L-NMMA is specifically due to inhibition of NO synthesis (data not shown). Figure 3C shows that HIF-1α expression is abolished in siRNA knockdown BICR6 cells. This is accompanied by a reduction in expression of HK-II, HO1, and MMP9, which are downstream targets of HIF-1α. Figure 4A shows that treatment with the NOS inhibitor L-NMMA abolished the stabilization of HIF-1α at 3% O₂ in the three cell types assayed. L-NMMA treatment did not, however, affect the stabilization of HIF-1α when the O₂ concentration was lowered to 0.5%, as shown in Fig. 4B.

**Constitutive NO-dependent stabilization of HIF-1α in tumor cells.** To test directly whether the effect of endogenous NO on HIF-1α stabilization was due to its ability to generate free radicals, the three cell lines were incubated at 3% O₂ in the absence or presence of antioxidants and HIF-1α protein concentrations in the nuclear extracts were measured by Western blot. Incubation of the three cell lines with 2.5 mmol/L NAC and 1 mmol/L l-ascorbic acid prevented the stabilization of HIF-1α at 3% O₂ (Fig. 5A). When the experiment was carried out at lower O₂ concentrations (0.5%), no significant effect was observed after pretreatment with antioxidants (Fig. 5B).

**Discussion**

We have observed a widespread distribution of HIF-1α in 24 of 29 human samples of oral squamous cell carcinoma. The widespread distribution indicates that HIF-1α stabilization is not exclusively associated with areas of potential low O₂ concentration. This is in agreement with previous observations (10, 11) showing a dual distribution pattern for HIF-1α, one distal from the blood vessels, suggesting that it was probably related to hypoxia, and another diffuse pattern independent of vessel proximity.

In the three cell lines used, we found stabilization of HIF-1α at O₂ concentrations well above those that would be considered hypoxic and, therefore, independent of PHD inhibition. In one cell line, HIF-1α was observed even at ambient O₂ concentration (21%). The mechanism for this accumulation is not clear; however, our experiments indicate that it is related to NO because inhibition of its generation by the NOS inhibitor L-NMMA prevented HIF-1α stabilization in all three cell lines. In our studies on malignant tissue we found that, of the 24 samples containing HIF-1α, both iNOS and eNOS were widely expressed in 14 cases, whereas the remaining 10 cases expressed eNOS alone. In the cancer cell lines, we found the three isoforms of NOS without any obvious difference in protein concentration. NOS has been widely observed in cancer (21, 22). Originally, iNOS, which is induced in pathophysiologic situations such as inflammation and tissue degeneration, was thought to be associated with cancer (23); however, the involvement of eNOS has also been suggested (24). In our current results, eNOS was present more often than iNOS in the tumor samples examined. It is not currently known why the expression of different NOS isoforms in cancer is not uniform.

The fact that the NO-dependent effect on HIF-1α stabilization in the three cell lines studied could be inhibited by NAC and l-ascorbic acid suggests that the mechanism is dependent on a free radical reaction, secondary to an interaction of NO with O₂ or O₂-derived species. Malignant tissue is known to be rich in free radicals (25), the origin of which has not been completely elucidated (26). Thus, NO generated from any isoform might be released into an environment that favors free radical reactions, leading to, among other things, the formation of peroxynitrite, a highly oxidant species that has also been detected in some cancers (27). Thus, it is

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**Figure 4. Effect of endogenous NO on the accumulation of HIF-1α in oral squamous carcinoma cells at low O₂ concentrations.** A, HIF-1α protein accumulation in the nuclear extracts of cells treated with (+) or without (−) 1 mmol/L L-NMMA, exposed to 3% O₂ for up to 8 hours. B, as (A), but with cells exposed to 0.5% O₂. Representative of results obtained in three separate experiments.

**Figure 5. Effect of antioxidants on the accumulation of HIF-1α in oral squamous carcinoma cells.** A, Western blot analysis showing the accumulation of HIF-1α in nuclear extracts from cells exposed to 3% O₂ for up to 8 hours in the absence (−) or presence (+) of antioxidants (NAC and l-ascorbic acid). B, as (A), but with BICR6 cells exposed to 0.5% O₂. Representative of those obtained in three separate experiments.
possible that the effects of NO in the cancer phenotype are more dependent on the environment in which it is produced than on its concentration or the specific isoform of NOS by which it is generated.

The stabilization of HIF-1α at a low O2 concentration (0.5%) was not affected by treatment with L-NMMA, suggesting that hypoxia rather than NO was responsible for this stabilization. This suggestion is supported by the fact that antioxidants also had no effect on the stabilization of HIF-1α at this low O2 concentration. This is in agreement with previous observations (28). The stabilization of HIF-1α in this condition is most likely to be due to inhibition of PHDs (29).

In view of the above, it is possible that accumulation of HIF-1α in cancer may be the result of a complex interaction of several factors, including hypoxia and NO, the combination of which we have previously shown to be highly synergistic in stabilizing HIF-1α (15). The profile of activities of these different factors may vary not only from cancer to cancer but also from one region of a cancer to another. Traditionally, tumor hypoxia has been associated with poor prognosis and resistance to treatment (30). Because the hypoxia-associated resistance to treatment is probably due, at least in part, not to the hypoxia itself but to the cellular defense mechanisms activated by it, any other factors leading to the same defense mechanisms, such as in this case the effect of NO on HIF-1α stabilization, are likely to reinforce the cancer phenotype. A corollary of our study is that inhibition of NO generation, or a decrease in free radical formation, may be useful adjuncts to other therapies in the management of cancer.

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