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Advances in Brief

Hypoxia Inducible Factor (HIF-1a and HIF-2a) Expression in Early Esophageal Cancer and Response to Photodynamic Therapy and Radiotherapy

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Abstract

Hypoxia inducible factor 1a and 2a (HIF-1a and HIF-2a) are key proteins regulating cellular response to hypoxia. Because the efficacy of photodynamic therapy (PDT) is dependent on the presence of oxygen, the assessment of HIF-1a and HIF-2a expression may be of value in predicting clinical response to PDT. Using recently produced MoAbs, we examined the expression of HIF1a and HIF2a in a series of 37 early-stage esophageal cancers treated with PDT and with additional radiotherapy in case of incomplete response after PDT. Strong expression of the HIF1a and of HIF2a proteins in all optical fields examined was noted in 51% and in 13% of cases, respectively. High expression was associated with a low complete response (CR) rate and with the absence of bcl-2 protein expression. On the contrary, bcl-2 expression was associated with a high CR rate. Combined analysis of HIF1a and bcl-2 protein expression revealed that of 16 cases with high HIF1a expression and the absence of bcl-2 reactivity, only 1 (7%) responded completely to PDT ($P = 0.007$). Bivariate analysis showed that HIF1a expression was independently related to response to PDT ($P = 0.04$; t ratio = 2.8), whereas bcl-2 approached significance ($P = 0.07$; t-ratio = 1.8). The final response to radiotherapy was high (70%) and independent of the HIF and bcl-2 status, which may be a result of reoxygenation after cellular depletion mediated by PDT. The present study suggests that assessment of HIF and of bcl-2 expression are important predictors of *in vivo* sensitivity to PDT. Modulation of PDT response with bioreductive drugs and/or drugs targeting bcl-2 (*i.e.*, taxanes) may prove of significant therapeutic importance in a subgroup of patients with high HIF expression.

Introduction

HIF1a and HIF2a[2] have been recognized as key proteins regulating the transcription of multiple genes related to erythropoiesis, angiogenesis, glycolysis, and vasodilation (reviewed in Refs. 1 and 2). After exposure of normal and cancer cells to hypoxia, a rapid increase of HIF1a and HIF2a heterodimerization with the HIF1b protein (ARNT) occurs, leading to increasing amounts of the HIF1 and HIF2 protein in the cytoplasm and in the nucleus (3). Although stabilization of HIF proteins may occur because of mutations or oncogene activation (4, 5), HIF overexpression in tumors is likely to reflect pronounced tumor hypoxia.

Tumor hypoxia is a well-recognized factor linked to poor response to RT, but it is also linked to a poor response to chemotherapy (reviewed in Ref. 6). Cellular hypoxia is known to hinder the efficacy of PDT (7, 8). Bioreductive drugs have been reported to enhance the efficacy of PDT (9). The recent development of monoclonal antibodies against HIF1a (10, 11) and HIF2a (11) that work on paraffin-embedded tissue allow the investigation of the pathogenetic and the predictive role HIF expression and, therefore, of the associated hypoxia in human carcinomas.

In the present study we provide clinical evidence that HIF expression is associated with resistance of esophageal cancer to PDT, whereas bcl-2 protein (a known target of PDT) expression favors complete response.

Materials and Methods

We recently reported long-term survival data from patients with early esophageal cancer, which was still inoperable for various reasons, treated with PDT (12). A complete response rate of 40% was noted as well as long-term local control and favorable overall survival. Additional RT for patients who showed an incomplete response to PDT increased the CR rate to 57–89% (depending upon the tumor stage) and yielded a 35% 5-year disease-free survival.

Archival biopsy material (before PDT) from 37 patients recruited in this study were retrieved. Among the 37 patients, 14 (38%) had carcinoma *in situ* (esophagus length involved, 1–3 cm), 19 (51%) had T1-stage tumor (invading the lamina propria or the submucosa) and 4 (11%) had superficial recurrent tumors in the anastomotic area after previous surgery. In all cases, the tumor extension was <3 cm. The follow-up period for this group of patients ranged from 3 to 90 months (median, 32 months).

The PDT protocol has been described in detail in a previous study (12). Briefly, hematoporphyrin derivative (5 mg/kg) was administered 48–72 h before laser treatment. For light irradiation, an argon dye laser (630 nm wavelength; 300–800 mW power; median 500 mW) was used. The estimated energy dose delivered was 300 J/cm (output fluence rate measured in air). The light was directed to the lesion through optical cylindrical fibers of 1–3 cm permitting 360° irradiation.

Repeat esophagoscopy was performed 40 days after PDT to assess response, which allowed direct examination and biopsies to be performed. CT scan of the chest was also performed. Response was judged as complete (CR; no evidence of the lesion by both endoscopy and CT as well as by negative histology), partial (PR; more than 50% shrinkage of the lesion by endoscopy and CT), or nonresponse (minimal response, stable or progressive disease). A second PDT was performed in cases of PR or nonresponse.

All patients with incomplete response to PDT underwent radical RT (64 Gy, standard fractionation, LINAC 6 MV X-rays), as described previously (12). Patients who showed CR (pathologically confirmed) after one or two courses of PDT were monitored by endoscopy every 3 months for the first year and every 4 months thereafter with no additional treatment. Patients also underwent CT of the thorax and upper abdomen as well as full blood counts and biochemical tests every 4 months. If recurrent disease was confirmed by endoscopy, patients were treated with RT (64 Gy of total dose, standard fractionation) as described previously (12).

Immunohistochemistry. The HIF1a and HIF2a proteins were detected using the ESEE 122 (IgG1 Mab; dilution 1:20) and the EP190b (IgG1 Mab,

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[2] The abbreviations used are: HIF, hypoxia inducible factor; PDT, photodynamic therapy; CR, complete response; CT, computed tomography; RT, radiotherapy; APAAP, alkaline phosphatase/anti-alkaline phosphatase.

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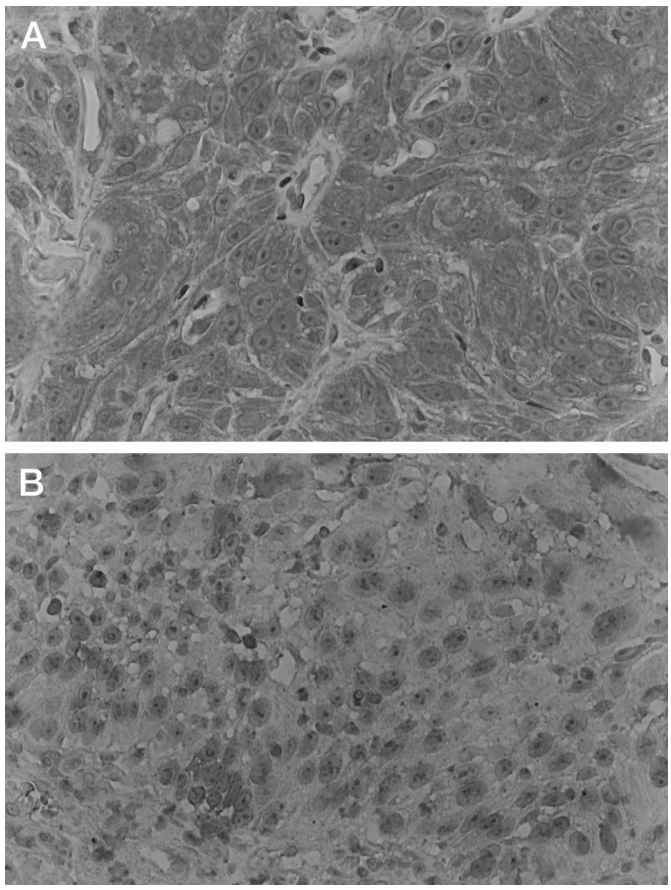

Fig. 1. Typical mixed nuclear/cytoplasmic immunostaining of esophageal cancer for HIF1a (Fig. 1*a*; ×400) and predominantly nuclear expression of HIF2a (Fig. 1*b*; ×400) protein.

neat; Ref. 11). Sections were deparaffinized, and staining was performed with the APAAP procedure. After microwaving (4 min × 2) the primary antibodies were applied at 4°C and incubated overnight. After washing in Tris-buffered saline, rabbit antimouse antibody 1:50 (v/v) was applied for 30 min and then application of mouse APAAP complex 1:1 (v/v) for 30 min. After washing in Tris-buffered saline, the last two steps were repeated for 10 min each. The color was developed by 20-min incubation with New Fuchsin solution. Breast cancer tissue sections with strong nuclear HIF1a and HIF2a expression were used as positive controls. Normal rabbit IgG was substituted for primary antibody as the negative control (same concentration as the test antibody). All optical fields were examined (3–7 × 200 fields/case).

The Bcl-2 protein expression was also assessed using the clone 100 MoAb (dilution 1:20; Dako, Denmark). The same APAAP technique was used for immunostaining. Lymph-node sections were used for positive control. bcl-2 cytoplasmic reactivity in >10% of cancer cells (all optical fields examined) was required to score a case as positive for bcl-2.

Statistical Analysis. The statistical analysis and generation of survival curves were performed using the GraphPad Prism 2.01 package (GraphPad, San Diego CA). Fisher's exact test was used to test associations between categorical variables. Survival curves were plotted using the method of Kaplan and Meier. *P*s of <0.05 were considered statistically significant.

Results and Discussion

HIF Patterns of Expression. The patterns of HIF1a and of HIF2a expression in cancer cells, when present, were mixed nuclear/cytoplasmic (Fig. 1). Adjacent normal mucosa showed a varying intensity of cytoplasmic staining (weak or even strong). Staining of tissue sections from normal esophagus showed that the normal epithelium does not express HIF proteins. These patterns of HIF1a and HIF2a expression in cancer cells have been reported by Talks *et al.* (11) and Zhong *et al.* (12), but normal mucosa was persistently negative in both studies. Our finding that HIF protein is expressed by the normal epithelium around the tumor, but not in normal esophagus, may show that hypoxia also concerns tissues around the cancerous lesions, probably as a result of the increased interstitial pressure exerted by the tumor or the accumulation of the metabolic products that affect the oxygen release by red cells and its consumption by tissues proximal to the tumor.

Strong expression of the HIF1a and of HIF2a proteins in all optical fields examined was noted in 19 of 37 (51%) and in 5 of 37 (13.5%) cases, respectively. These cases were considered as bearing high HIF reactivity. The remaining cases, showing very weak or no HIF expression, were considered as bearing low HIF reactivity. All cases with high HIF2a reactivity also had HIF1a expression.

HIF Expression and Response to PDT. There was no difference, in terms of response, between *in situ* and T1-staged cases. Table 1 shows the response rate to PDT and to RT according to HIF expression. A statistically significant association of high HIF1a with a poor response to PDT was noted ($P = 0.03$). None of the five cases with HIF2a had a complete response to PDT. This is in accordance with *in vitro* studies showing that the presence of oxygen is essential for the photodynamic reaction to occur (7, 8); low oxygen amounts relate to impaired PDT efficacy and incomplete response (13).

Combined HIF/bcl-2 Analysis. The bcl-2 protein expression also has been assessed in the same material. bcl-2 is a dimeric protein located on the mitochondrial walls and antagonizes the release of the mitochondrial cytochrome *c*, which induces cellular apoptosis immediately after its entrance into the cytoplasm (14, 15). Mitochondria seem to be a principal target of PDT (16). In a recent study, Kim *et al.* (17) showed that PDT causes a selective destruction of bcl-2, leaving bax unaffected. In that way the bcl-2/bax dimer is decomposed and cytochrome *c* is released in the cytoplasm resulting in cellular apoptosis.

In the present study, bcl-2 protein expression was significantly associated with a better response to PDT. The CR rate in bcl-2-positive cases was 7 of 11 (64%) *versus* 6 of 26 (23%) in bcl-2 negative cases ($P = 0.05$). Combined analysis of HIF1a and bcl-2 protein expression revealed that the absence of HIF1a expression was linked to a good CR rate whether cases expressed bcl-2 (CR, 5 of 8) or not (CR rate, 5 of 10). Of 16 cases with high HIF1a expression and the absence of bcl-2 reactivity, only one (7%) showed CR *versus* 12 of 21 (57%) in the remaining cases ($P = 0.007$). Bivariate analysis showed that HIF1a expression was independently related to incom-

Table 1 *HIF1a, HIF2a, bcl-2 and response of esophageal cancer to PDT*

	Response to PDT			
	CR	PR	MR/NR[a]	P^b
HIF1a				
Low	10	6	2	0.04
High	4	11	5	
HIF2a				
Low	14	12	7	0.13
High	0	5	0	
Bcl-2				
Low	6	14	6	0.02
High	7	3	1	
HIF1a/bcl-2				
High/low	1	10	5	0.007
All other	12	7	2	
HIF2a/bcl-2				
High/low	0	5	0	0.13
All other	13	12	7	

[a] MR/NR, minimal or no response.
[b] *P* values refer to CR *versus* all other responses.

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plete response after PDT ($P = 0.04$; t ratio = 2.8), whereas bcl-2 correlation with CR approached significance ($P = 0.07$; t ratio = 1.8).

An inverse association of HIF1a expression with bcl-2 expression was noted (8 of 18 cases with low HIF1a expression expressed bcl-2 *versus* 3 of 19 of cases with high HIF1a expression; $P = 0.07$). This inverse association has been also reported by Zhong *et al.* (10), in an analysis that included different tumor types, and also in a study of ours on non-small cell lung cancer.[3] Because of this inverse association, only 3 of 33 esophageal carcinomas bore the "high HIF1a/high bcl-2" phenotype. Two of these cases, however, had a complete response. All five cases with HIF2a high reactivity were negative for bcl-2.

HIF Expression and Response to Additional RT. The response rate to additional RT of lesions with incomplete response to PDT was not related to HIF expression ($P = 0.35$; data not shown) or to bcl-2 expression ($P = 0.28$; data not shown). PDT rapidly consumes the available tissue oxygen, and the damage exerted at the vascular level (18) may well render euoxic tumors quite hypoxic. However, the CR rate of remnant lesions to RT was high (17 of 24; 70%). This could be a result of the very low tumor burden of the lesions included in the study, which may minimize the clinical importance of the increased hypoxia levels induced by PDT. Furthermore, a recent *in vitro* study suggests that although the oxygen tension decreases during PDT, it is restored to the preirradiation levels shortly afterward (19). In that way, the initial PDT-mediated hypoxic stress may not affect the RT results. On the contrary, reoxygenation may rapidly appear within some days as a result of the depopulation induced by PDT, and the tumor oxygenation status may become better than the pretreatment one, which may explain the high CR rate of the residual lesions after RT.

HIF, bcl-2 Expression, and Survival. Survival analysis showed that, despite the association of HIF1a and of bcl-2 with response to PDT, none of the proteins were significantly associated with the local relapse-free survival ($P = 0.14$ and $P = 0.10$, respectively). A marginal association with overall survival was noted ($P = 0.08$ and $P = 0.03$, respectively). This can be explained by the fact that the final prognosis depended on the outcome after combined PDT/RT, and none of the proteins were associated with the final response to the PDT/RT regimen. Complete responders after PDT/RT had a significantly better overall and local relapse-free survival times compared with patients with incomplete response ($P < 0.001$; data not shown). The low number of patients included in the study may also account for the failure to show a clear association of HIF with survival parameters. In a recent study, Birner *et al.* (20) found a strong association of HIF1a expression with poor outcome in early stage pT1b cervical cancer. Similarly, we found a significant association of HIF1a/HIF2a expression with poor survival in operable (stage T1,2-N0,1) non-small cell lung cancer.[3]

Conclusions and Implications

It is concluded that HIF expression is a common event in esophageal cancer, and that it is related to impaired efficacy of PDT to eradicate the disease. HIFs are key proteins induced by hypoxia, and HIF-expressing tumors should be intensively hypoxic, which accounts for the high incomplete response rate following PDT and is in accordance with experimental studies suggesting a close link between oxygenation and PDT-induced cellular damage. Bioreductive drugs enhance the cytotoxicity of PDT *in vitro* and may be of clinical value in a subset of patients with HIF-expressing lesions. However, we should stress that HIF stabilization may also be a result of the mutations of a variety of genes (4, 5, 21). As no measurements of tumor pO2 were made in the present study, an eventual role of such genetic events in guiding response to PDT cannot be excluded. bcl-2 protein is a well-established target of PDT and its expression in esophageal cancer correlates with favorable response. Although, in the present study, hypoxia seems to be a more potent factor related to the response to PDT, modulation of the bcl-2/bax equilibrium before PDT may be of clinical value. Taxanes or other agents involved in the bcl-2 phosphorylation (22) may enhance the low magnitude of the bcl-2 damage that PDT produces under hypoxic conditions.

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