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Hypoxic Microenvironmental Control of Stress Protein and Erythropoietin Gene Expression

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The presence of hypoxic cells in solid tumors has long been considered a problem in cancer treatment such as in radiation therapy or treatment with some anticancer drugs. It has been suggested that hypoxic cells are involved in the development of a more aggressive phenotype and contribute to metastasis. In this study, as an attempt to understand how tumor cells adapt to hypoxic stress, we investigated the regulation of the hypoxia-induced expression of proteins that control essential processes of tumor cell survival and angiogenesis. We first examined whether hypoxia induces stress protein gene expression of murine solid tumor RIF cells. We also examined hypoxia-induced changes in angiogenic gene expression in these cells. Finally, we investigated the association of the elevated levels of stress proteins with the regulation of hypoxia-induced angiogenic gene expression. Results demonstrated that hypoxia induced the expression of the erythropoietin (*EPO*) gene and at least two major members of stress proteins, heat shock protein 70 (HSP70) and 25 (HSP25) in RIF tumor cells. Evidence that the expression of *EPO* gene was greatly potentiated in TR cells suggested that the elevated levels of HSPs may play an important role in the regulation of the hypoxia-induced *EPO* gene expression. One of the RIF variant cell lines, TR, displays elevated levels of HSPs constitutively. Taken together, our results suggest that a hypoxic tumor microenvironment may promote the survival and malignant progression of the tumor cells by temporarily increasing the level of stress proteins and expressing angiogenic genes. We suspect that stress proteins may be associated with the increase of the angiogenic potential of tumor cells under hypoxia.

Keywords: Angiogenesis, Erythropoietin, Heat shock protein, Hypoxia, Solid tumor.

Introduction

Many malignant tumors contain significant fractions of hypoxic cells as a consequence of their inadequate vascular networks (Moulder and Rockwell, 1987; Vaupel, 1989). Reoxygenation of the hypoxic cells occurs during unperturbed tumor growth due to reopening of the temporarily closed or blocked vessels (Brown, 1979) and during fractionated radiation therapy due to the radiation-induced tumor cell inactivation (Kallman, 1972). Fluctuations in red cell flux in tumor microvessels can also lead to the generation of hypoxic microenvironments in tumor parenchyma (Kimura *et al.*, 1996).

Hypoxic tumor cells are more resistant to the effects of radiotherapy and many conventional chemotherapeutic agents than their normoxic counterparts (Teicher *et al.*, 1981; Gatenby *et al.*, 1988; Teicher *et al.*, 1994). Hypoxic tumor cells may also be involved in a disease relapse and metastasis (Schwickert *et al.*, 1995; Brizel *et al.*, 1996). Consequently, the presence of hypoxic cells in solid tumors has long been considered a problem in cancer treatment. Three general strategies have been actively explored to overcome the problem: oxygenating the tumor, sensitizing the hypoxic cells to radiation or chemotherapy, or killing the hypoxic cells with hypoxic cell cytotoxins (Brown and Koong, 1991). A new and as yet unexplored avenue to overcome the problem of tumor hypoxia would be to inhibit synthesis of hypoxia-induced proteins that are required for tumor cell survival and angiogenesis.

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Although the mechanisms by which tumor cells adapt to hypoxic stress are not completely understood, hypoxia is known to alter the gene expression pattern of tumor cells significantly (Brown and Giaccia, 1994). Investigators in the field have suggested that hypoxia elicits a stress response and the synthesis of stress proteins both *in vitro* and *in vivo* (Guttman *et al.*, 1980; Heacock and Sutherland, 1990; Iwaki *et al.*, 1993). It is well known that one of the major functions of stress proteins is to protect cells against various cytotoxic conditions. The expression of 78 kDa glucose-regulated stress protein (GRP78) has also been implicated in enhanced tumor cell survival against hypoxic stress (Koong *et al.*, 1994a; 1994b).

The stress protein family was also linked to the development of a drug resistance in tumor cells (Wilson and Sutherland, 1989; Murphy *et al.*, 1991). The effect of the overexpression of a member of the stress protein family, HSP27, in human testis tumor cells, on heat shock as well as on drug treatment was reported (Richards *et al.*, 1996). Moreover, some of these stress proteins have been implicated in hypoxia-induced radiation resistance (Danielson *et al.*, 1997). These results support the hypothesis that the expression of stress proteins in hypoxic tumor cells contributes to the development of a more aggressive phenotype as well as to the generally enhanced tumor cell survival after radiation or chemotherapy. It appears that slightly different stress proteins are induced depending on the cell types and their microenvironment.

In order for hypoxic tumor cells to progress and acquire a metastatic potential, one of the essential processes is the development of new blood vessels, i.e., angiogenesis. A number of growth factors involved in angiogenesis were shown to be induced by hypoxia. These include erythropoietin (*EPO*) (Wang and Semenza, 1993), vascular endothelial growth factor (*VEGF*) (Stein *et al.*, 1995), and transforming growth factor-$\beta 1$ (*TGF*-$\beta 1$) (Brown *et al.*, 1997). Some of these angiogenic factors were also implicated in tumor malignancy (Iruela-Arispe and Sage, 1993; Abu-Jawdeh *et al.*, 1996). The concerted induction of stress protein and angiogenic factor gene expression by hypoxia appears to help tumor cells adapt to hypoxic stress and to progress to a more malignant phenotype.

In this study, we first examined the expression profiles of stress proteins in hypoxic solid tumor RIF cells. We also examined the regulation of hypoxia-induced angiogenic factor gene expression. The expressions of the *EPO*, *VEGF*, and *TGF*-$\beta 1$ genes, in particular, were examined in these cells. Finally, we investigated a possible role for stress proteins on the regulation of hypoxia-induced angiogenic factor gene expression.

Materials and Methods

Cells and culture conditions The RIF cell line was derived from a radiation-induced fibrosarcoma (Twentyman *et al.*, 1980) and the TR line was developed from the RIF (Hahn and van Kersen, 1988). Both RIF and TR cells were cultured in RPMI 1640 (GIBCO-BRL, Richmond, USA), supplemented with 10% fetal calf serum, 200 mg/l streptomycin sulfate, and 190,000 U/l penicillin G potassium at 37°C in an atmosphere of 5% CO_2–95% air. Exponentially growing cultures were used for all experiments.

Hypoxia Cells were subjected to hypoxia by combining oxygen-glucose deprivation at 37°C in a hypoxic chamber (Forma Scientific, Marietta, USA). Briefly, before each experiment, the medium was pre-equilibrated with a hypoxic gas mixture containing 5% CO_2, 85% N_2, and 10% H_2 at 37°C. Cells were deprived of oxygen and glucose by triple exchange of the culture medium with deoxygenated, glucose-free medium (GIBCO-BRL, Richmond, USA). The oxygen concentrations in the hypoxic chamber and in the exposure medium were monitored by an oxygen indicator (Forma Scientific, Marietta, USA). The oxygen tension in the chamber was maintained at 0.02%. Hypoxia was terminated by exchanging glucose-containing medium and returning cells to the normoxic incubator.

Measurement of total protein synthesis rate The effect of hypoxia on total protein synthesis was determined by measuring the uptake of [^{35}S] labeled amino acids as described previously (Anderson *et al.*, 1989). Briefly, cells were exposed to hypoxia for increasing time intervals. At the end of the hypoxic treatment, cells were exposed to medium containing [^{35}S]methionine (specific activity > 140 Ci/mM, Amersham) for 30 min at 37°C. Aliquots were taken for the measurement of radioactivity and the analysis of the proteins being synthesized (Peterson and Mitchell, 1981). The rate of protein synthesis was calculated as the radioactivity incorporated per μg protein.

Western blot analysis Equal amounts of proteins were analyzed by SDS-polyacrylamide gel electrophoresis in duplicate on a 13% SDS-polyacrylamide gel (Laemmli, 1970). Protein concentration was measured by the method of Lowry *et al.* (1951). After electrophoresis, one gel was stained with Coomassie blue, and the second gel was transferred to a nitrocellulose membrane in a Trans-Blot apparatus (BioRad, Richmond, USA). The membrane was incubated with anti-HSP70 Ab or anti-HSP25 Ab (StressGen, Victoria, Canada). After washing the blots to remove unbound antibodies, the blots were visualized with the ECL system (Amersham, Buckinghamshire, England).

RNA isolation and RT-PCR analysis Total cellular RNA was extracted by the modified single step guanidium isothiocyanate lysis method (Chomczynski and Sacchi, 1987). For reverse transcription (RT) coupled with polymerase chain reaction (PCR), one μg of total RNA was reverse transcribed at 37°C using random hexamer primers and MMLV (Moloney murine leukemia virus) reverse transcriptase in a 20 μl reaction mixture. The RT-PCR was carried out by adding a mix containing oligonucleotide primers (20 pmole each), dNTP (1.25 mM each), 10× PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin) and AmpliTaq DNA polymerase (Perkin Elmer, Norwalk, USA) to 2 μl of each reverse transcription reaction mixture. Locations and the sequences of the PCR primer pairs used for the amplification of *hsp70* (Hunt and Calderwood, 1990) and *hsp25* (Gaestel *et al.*, 1993) genes are shown in Table 1. To serve as an

internal control as well as to monitor the consistency of the PCR reaction, primers for the *hprt* gene (Konecki *et al.*, 1982) were also designed. Table 2 shows the locations and sequences of the PCR primer pairs to amplify angiogenic factors, *EPO* (McDonald *et al.*, 1986), *VEGF* (Claffey *et al.*, 1992), and *TGF-β1* (Derynck *et al.*, 1986). Amplification was carried out in a Perkin Elmer thermal cycler (Perkin Elmer, Norwalk, USA). The amplified products were separated by electrophoresis on 1.5% agarose gels, stained by ethidium bromide and photographed under UV illumination. The intensity of each band was quantitated by using a densitometer (Molecular Dynamics, Sunnyvale, USA).

Results

Inhibition of protein synthesis by hypoxia Hypoxic microenvironments have been shown to cause various intracellular physiological changes. In order to investigate the hypoxic tumor cell biology *in vitro*, we examined the effect of hypoxia on the rate of total protein synthesis in solid tumor RIF cells. The cells were subjected to hypoxia for increasing time intervals. At the end of the hypoxic exposure, the rate of total protein synthesis was determined as a function of time. As shown in Fig. 1, a significant inhibition of the protein synthesis was observed after 2 h exposure to hypoxia such that the hypoxic protein synthesis rate was about 67% of the normoxic control cells. This level of inhibition persisted until 8 h exposure to hypoxia. However, a decrease in cell number due to the hypoxia-induced cell death was observed after 8 h exposure to hypoxia. Since cell survival and morphology were nearly normal up to 4 h exposure to hypoxia, this exposure time was chosen to be used throughout the current study.

Increased *hsp70* and *hsp25* gene expression following 4 h hypoxia To investigate how tumor cells adapt to hypoxic stress, we examined if hypoxia induced the expression of stress proteins. Among the members of the stress protein family, the *hsp70* and *hsp25* gene inductions appeared to be the most pronounced following 4 h exposure to hypoxia (Fig. 2). We examined changes in the levels of *hsp70* and *hsp25* mRNAs by RT-PCR analysis. The result demonstrated the accumulation of *hsp70* and *hsp25* mRNAs upon induction. We found a maximum accumulation of *hsp70* mRNA immediately after hypoxia before the onset of reoxygenation, indicating that the induction of *hsp70* mRNA gene expression occurred

Table 1. Locations and nucleotide sequences of the PCR pairs for *hsp70* and *hsp25* mRNAs.

Gene	Nucleotide sequences	Product size (bp)	Locations	References
hsp70	5′-CCCGCGTGCTAATGGTGGAC-3′ 5′-TTCCGTTTCCAGCCCCCAATC-3′	558	435–455 993–974	Hunt and Calerwood, 1990
hsp25	5′-GCCCGAGCTGGGAACCATTC-3′ 5′-CGCCTTCCTTGGTCTTCACT-3′	304	793–813 1097–1077	Gaestel *et al.*, 1993
hprt	5′-GTTGGATACAGGCCAGACTTTGTTG-3′ 5′-GATTCAACTTGCGCTCATCTTAGGC-3′	162	601–629 763–739	Konecki *et al.*, 1982

To amplify *hsp70* and *hsp25* mRNAs, the cycles of heating (94°C, 1.5 min), annealing (55°C, 1.5 min), and polymerization (72°C, 1.5 min) were continued for 35 cycles. To amplify *hprt* mRNA, the cycles of heating (94°C, 1.5 min), annealing (55°C, 1min), and polymerization (72°C, 1 min) were continued for 30 cycles.

Table 2. Locations and nucleotide sequences of the PCR pairs for angiogenic genes.

Gene	Nucleotide sequences	Product size (bp)	Locations	References
EPO	5′-AGCCCTGCGTCTAATGTTTC-3′ 5′-CGACCACCAGAGACCCTTCA-3′	537	2317–2336 2854–2835	McDonald *et al.*, 1986
VEGF	5′-TGCACTGGACCCTGGCTTTA-3′ 5′-TTTGCAGGAACATTTACACG-3′	472	109–128 581–562	Claffey *et al.*, 1992
TGF-β1	5′-CCCGCGTGCTAATGGTGGAC-3′ 5′-AGCGCCCGGGTTGTGTTGGT-3′	724	678–692 1402–1383	Derynck *et al.*, 1986
hprt	5′-GTTGGATACAGGCCAGACTTTGTTG-3′ 5′-GATTCAACTTGCGCTCATCTTAGGC-3′	162	601–629 763–739	Konecki *et al.*, 1982

To amplify *VEGF* and *TGF-β1* mRNAs, the cycles of heating (94°C, 1.5 min), annealing (55°C, 1.5 min), and polymerization (72°C, 1.5 min) were continued for 35 cycles. To amplify *EPO* and *hprt* mRNAs, 40 and 30 cycles were employed, respectively.

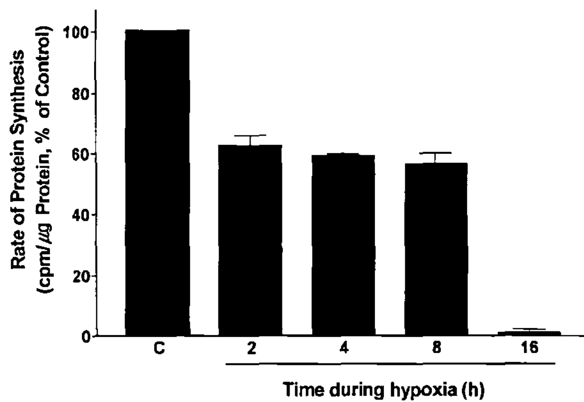

Fig. 1. Effects of hypoxia on total protein synthesis rate. Cells were subjected to hypoxia for increasing time intervals. The rate of the total protein synthesis is shown as percentage of the control rate, normoxic value as a function of time. C, control normoxic RIF cells.

during the hypoxic exposure (Fig. 2A). The level of *hsp70* mRNA was three times as much as that of normoxic control cells at the end of the exposure. The basal expression of *hsp70* mRNA was observed in control cells. The expression patterns of *hsp25* mRNA was somewhat different from those of *hsp70* mRNA (Fig. 2B). Unlike *hsp70* mRNA, the presence of *hsp25* mRNA was not found in control cells. The maximum level of *hsp25* mRNA accumulation was observed after 4 to 8 h following hypoxia, which was much later than that of *hsp70* mRNA. The levels of both mRNAs, however, declined after 8 h following hypoxia.

The accumulation of HSP70 and HSP25 proteins was also analyzed by Western blot analysis. The results revealed that, although *hsp70* mRNA levels were already high immediately after the hypoxic exposure, the accumulation of HSP70 protein was detected after 4 to 16 h following hypoxia (Fig. 3A). The mRNA levels of *hsp25* were generally well correlated with those of protein (Fig. 3B). HSP70 and HSP25 protein levels remained elevated until 16 h following hypoxia. No basal expressions of HSP70 or HSP25 were detected in RIF cells.

Effect of 4 h hypoxia on the regulation of angiogenic factors Since the development of the angiogenic potential in hypoxic tumor cells has been suggested, we examined whether the 4 h hypoxia induces angiogenic factor gene expression in solid tumor RIF cells. Hypoxia-induced changes in *EPO*, *VEGF*, and *TGF-β1* mRNAs were analyzed by RT-PCR analysis following 4 h hypoxia. As shown in Fig. 4, our results demonstrated that the basal levels of *VEGF* and *TGF-β1* mRNAs were high in normoxic control RIF cells. There might be marginal increases in *VEGF* and *TGF-β1* mRNAs (if any) in RIF cells following hypoxia, however, no significant increase in either was observed. Unlike *VEGF* and *TGF-β1*

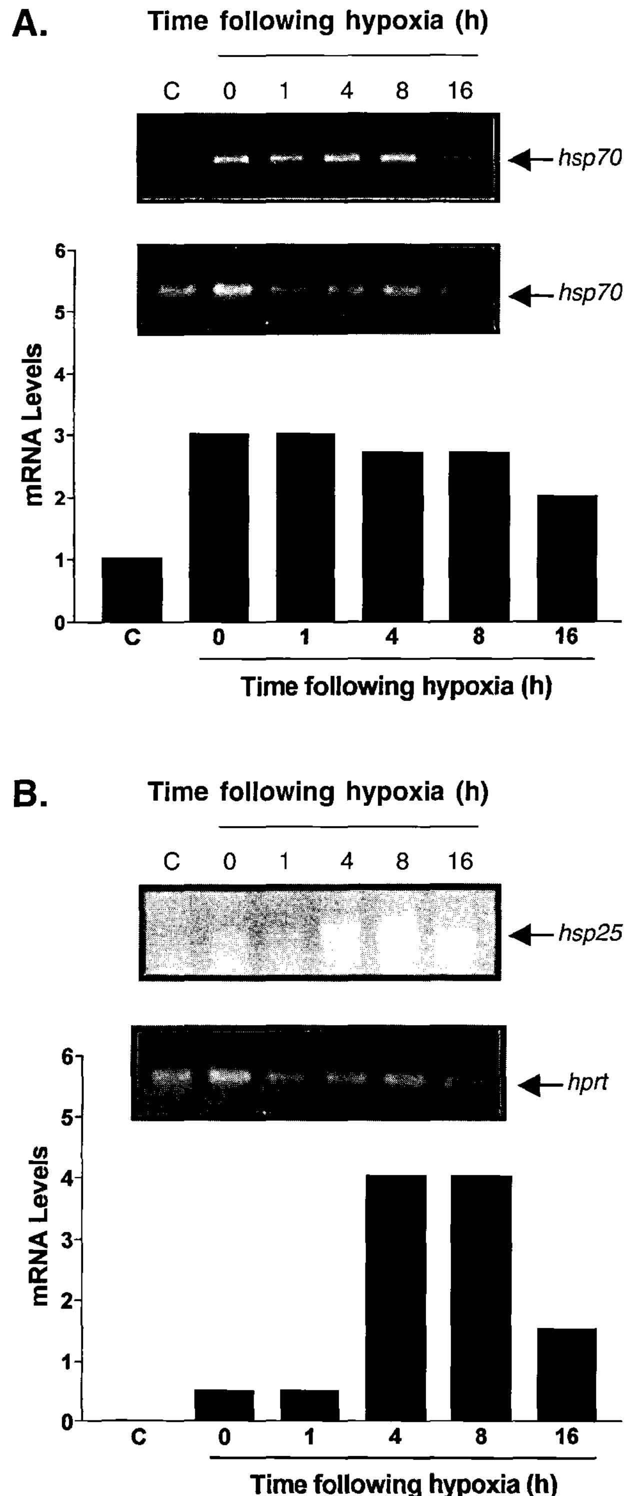

Fig. 2. RT-PCR analysis of *hsp70* and *hsp25* mRNAs following hypoxia. Total RNA was isolated from RIF cells after 0, 1, 4, 8, or 16 h following 4 h hypoxia. One μg of total RNA from each sample was subjected to RT-PCR analysis using an oligonucleotide pair specific to either *hsp70* (A) or *hsp25* mRNA (B). The *hsp70* or *hsp25* RT-PCR bands were quantitated and normalized to the values of *hprt* mRNA internal control bands. C, control normoxic RIF cells.

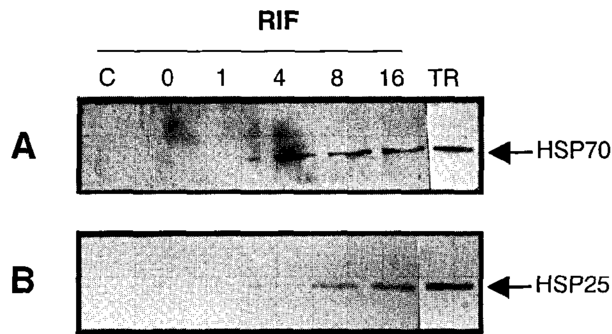

Fig. 3. Western blot analysis of HSP70 and HSP25 proteins following hypoxia. RIF cell were extracts were obtained after 0, 1, 4, 8, or 16 h following 4 h hypoxia. The samples were subjected to Western blot analysis using HSP70 Ab (A) or HSP25 Ab (B). The positions of the proteins are indicated on the right. C, control normoxic RIF. Basal level expressions of the of HSP70 and HSP25 proteins in TR cells are shown for comparison.

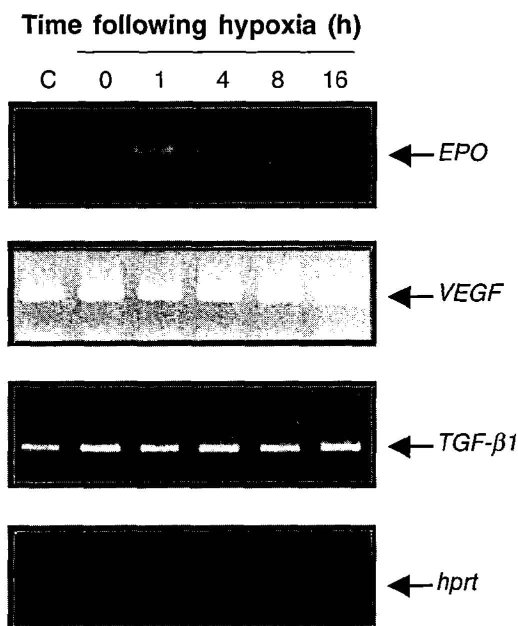

Fig. 4. RT-PCR analysis of angiogenic factor gene expression following hypoxia. Total RNA was isolated from RIF cells after 0, 1, 4, 8, or 16 h following 4 h hypoxia. One μg of total RNA from each sample was subjected to RT-PCR analysis using an oligonucleotide pair specific to *EPO*, *VEGR*, or *TGF-β1* mRNAs. The primer pair for *hprt* was used as an internal control.

mRNAs, the presence of the *EPO* mRNA was not detected in normoxic control cells. However, a significant accumulation of *EPO* mRNA was found following hypoxia in these cells, where a maximum accumulation was observed after 1 h. The levels gradually declined after 1 h and returned to that of the control after 16 h following hypoxia.

Coordinated expression of HSP and *EPO* mRNA in RIF and TR cells Having found the concerted regulation for the gene expression of stress proteins and *EPO*, we examined a possible role of the stress proteins in the regulation of *EPO* mRNA expression. To answer this question, a variant of the RIF cell line, TR, was employed. It was previously reported that the major stress proteins are constitutively expressed at elevated levels in these cells (Anderson *et al.*, 1989; Park *et al.*, 1993). The basal expression levels of HSP70 and HSP25 proteins in TR cells are shown in comparison with hypoxia-induced accumulation of these proteins in RIF cells in Fig. 3. We subjected both RIF and TR cells to hypoxia and compared the induction profiles of *EPO* mRNAs following hypoxia. As shown in Fig. 5, we found generally higher levels of *EPO* mRNA expression in TR cells than in parental RIF cells. In addition, while the maximum accumulation of *EPO* mRNA was observed after 1 h following hypoxia in RIF cells, a significant accumulation of the mRNA was apparent immediately after hypoxia in TR cells. The elevated levels of *EPO* mRNAs persisted much longer in TR cells than in RIF cells and started declining at 8 h following hypoxia. A decrease of *EPO* mRNA levels was apparent in RIF cells after 1 h following hypoxia. The presence of *EPO* mRNAs was not detected in either normoxic control RIF or TR cells.

Discussion

The current study aimed to investigate how tumor cells adapt to hypoxic stress and develop into more resistant phenotypes. Hypoxia inevitably encompasses the shortage of both oxygen and nutrient supplies. Therefore, we employed a combined oxygen-glucose deprivation *in vitro* to mimic a hypoxic tumor microenvironment. Our results showed that hypoxia resulted in the accumulation of the two major members of stress proteins, HSP70 and HSP25, in solid tumor RIF cells. Our data also demonstrated that hypoxia induced the accumulation of erythropoietin (*EPO*) mRNA in these cells. Evidence that the expression of *EPO* mRNA is greatly potentiated in TR cells suggested that the elevated levels of *hsp* gene expression may play a role in the regulation of the hypoxia-induced *EPO* mRNA expression.

It is generally believed that one of the major functions of stress proteins is to protect cells from ill effects of various stress conditions. Cells with an elevated expression of stress proteins, induced by either mild hyperthermia or gene transfection, were shown to be resistant to apoptotic cell death (Samali and Cotter, 1996). Overexpression of stress proteins was suggested to be linked to the resistances to heat and chemotherapeutic drugs (Richards *et al.*, 1996). Therefore, it is not surprising that HSP70 and HSP25 were accumulated in solid tumor RIF cells following hypoxia. The accumulation was observed at both the transcriptional and translational levels. It is likely that these proteins

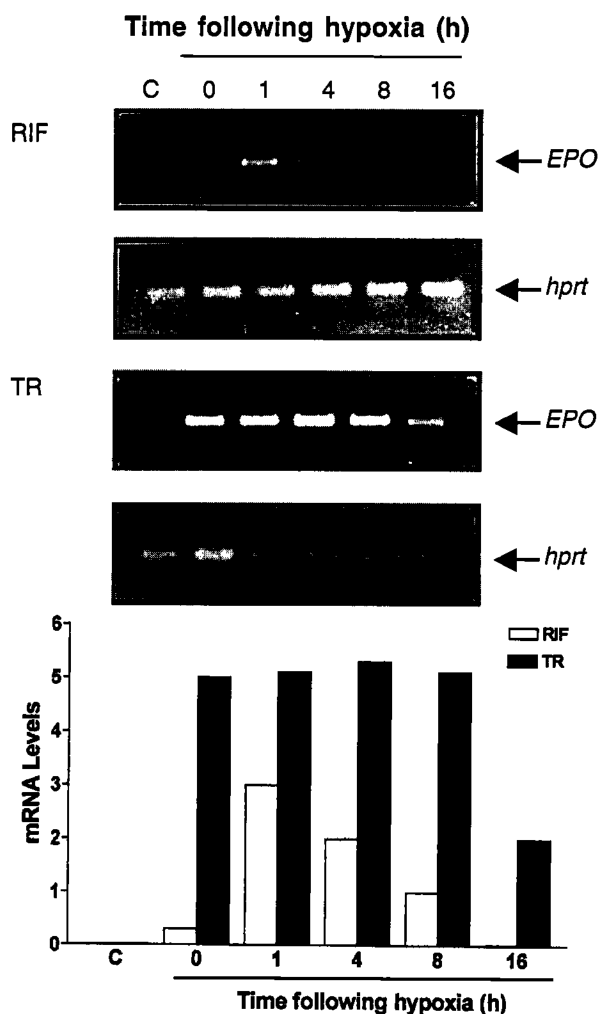

Fig. 5. Analysis of *EPO* gene expression in RIF and TR cells following hypoxia. Total RNA was isolated from RIF and TR cells after 0, 1, 4, 8, or 16 h following 4 h hypoxia. One μg of total RNA from each sample was subjected to RT-PCR analysis using an oligonucleotide pair specific to *EPO* mRNA. The RT-PCR bands from RIF and TR cells were quantitated and normalized to the values of the respective *hprt* internal control bands. C, control normoxic RIF or TR cells.

contribute to RIF tumor cells' ability to adapt to hypoxic stress and to develop more resistant phenotypes.

Regulation of *EPO* mRNA by cellular O_2 tension is known to represent a homeostatic mechanism for maintaining tissue oxygenation (Wang and Semenza, 1993). This adaptive response takes place both under normal physiology, upon increase in tissue mass from work overload, and under pathophysiological conditions such as tumor hypoxia generated as a result of functional impairment of blood vessels. Our finding that *EPO* mRNA was up-regulated in hypoxic solid tumor cells is consistent with the view that tumor cells sense hypoxic microenvironments and respond by eliciting compensatory angiogenesis, thereby matching supply to demand. Recently, the increased *EPO* has also been suggested to limit hypoxia-induced damage to neurons (Nakamura *et al.*, 1998).

Interestingly, our data indicated that the presence of the elevated levels of HSPs may be associated with the regulation of the *EPO* mRNA gene expression. Considering the fact that a variety of mRNAs encoding soluble growth factors are intrinsically unstable, it is plausible to hypothesize that the elevated levels of HSPs contribute to *EPO* mRNA stabilization. We are currently testing this hypothesis by determining the half life of *EPO* mRNA after treating cells with a transcriptional inhibitor, actinomycin D. We suspect that the increased *EPO* mRNA accumulation following hypoxia results from the increased transcription as well as mRNA stabilization. Currently, many attempts are being made to overcome the problem of tumor hypoxia by inhibiting synthesis of hypoxia-induced proteins that are required for tumor cell survival and angiogenesis. We believe our finding can be exploited to develop molecular strategies for the hypoxic solid tumor therapy.

Taken together, our results suggest that hypoxia followed by reoxygenation might promote the survival and malignant progression of tumors by temporarily increasing the HSP and angiogenic gene expressions, respectively. We suspect that HSPs may also have a role in the increase of the angiogenic and metastatic potential of tumor cells by potentiating the hypoxia-induced angiogenic gene expression.

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