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TNFα RECEPTOR1 DRIVES HYPOXIA-PROMOTED INVASIVENESS OF HUMAN MELANOMA CELLS

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***Aim*: Oxygen deprivation leading to hypoxia represents a common feature of advanced solid tumors, able to control several aspects of tumor progression. Indeed, ability to respond to changes in oxygen partial pressure represents a hallmark of malignant cells. Aim of this study is to disclose new pathway of hypoxia-induced tumor cell invasion. *Materials and Methods*: Hs294T human melanoma cells were grown in a gas mixture containing 0.3% O_2 and used to evaluate invasion on Matrigel-coated polycarbonate filters mounted in Boyden's chambers, MMP release and expression of inflammatory receptors and their ligands. *Results*: We demonstrate that hypoxia promotes the expression of TNFα receptor 1 (TNFαR1) able to drive a higher ability to penetrate Matrigel-coated filters of Hs294T human melanoma cells, an effect does not mediated by hypoxia-inducible factor (HIF)-1α. *Conclusion*: Expression of inflammatory cytokine receptors in hypoxic human melanoma cells might provide a new target for improving strategies against local and distant tumor cell diffusion.**

***Key Words*: TNFα receptor 1, Hs294T human melanoma cells, hypoxia, cell invasiveness, metalloproteinase 2 and 9.**

A critical reduction in oxygen availability is a common feature of many conditions including cancer. Hypoxia characterizes the environment of many solid tumors and has been shown to affect many biological properties implicated in tumor growth and metastasis, e.g. metabolic switch from oxidative to glycolytic metabolism, production of vascular endothelial growth factors and protease activities [1–3]. Hypoxic tumor cells undergoing a glycolytic switch from aerobic to anaerobic metabolism, may maintain activities under conditions of a reduced concentration of oxygen. However, in some circumstances anaerobic metabolism might be insufficient to meet the energy requirements, thus additional adaptations are required. Tumor cells that have the capacity to exhibit additional changes possess a survival advantage. In particular, hypoxic tumor cells may release angiogenic factors that stimulate the formation of new blood vessels in order to increase oxygen supply, glucose and nutrients to meet the metabolic demands of proliferating tumor cells [4]. The main part of these adaptations depends on hypoxia-inducible factor (HIF)-1α signalling. Several studies indicate that HIF-1α is overexpressed in primary and metastatic human tumors and associated with glucose metabolism, tumor angiogenesis and aggressiveness [5–7]. HIF-1α has been shown to promote tumor cell migration stimulating the receptor of tyrosine kinase c-Met [8, 9] and by up-regulation of pivotal controllers of protease system, such as urokinase plasminogen activator receptor (uPAR) and matrix metalloprotease 2 (MMP-2) [5]. Another marker of invasion and crucial for epithelial-to-mesenchymal transition (EMT) of tumor cells is loss of E-cadherin expression: HIF-1α may repress E-cadherin by a mechanism involving Snail, Slug labile nuclear factors [10].

In the present study, we demonstrate that the enhanced invasiveness acquired by Hs294T human melanoma cells exposed to hypoxia relates to TNFαR1 up-regulation, and HIF-1α is not critical for mediating this effect.

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Abbreviations used: HIF-1α – hypoxia-inducible factor-1α; MMP-2 – matrix metalloprotease 2; MMP-9 – matrix metalloprotease 9; TNFαR1 – TNFα receptor 1; uPAR – urokinase plasminogen activator receptor.

MATERIALS AND METHODS

Cell lines and culture conditions. Hs294T human melanoma cells used in this study were obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA). Melanoma cells were grown in Dulbecco's modified Eagle medium containing 4500 mg/L glucose (DMEM 4500, GIBCO) supplemented with 10% foetal calf serum (FCS) (GIBCO), at 37 °C in a humidified atmosphere containing 5% CO_2. 5.0×10^5 cells were seeded in 100 mm Sarstedt dishes and propagated every three days by incubation with a trypsin-EDTA solution (GIBCO). Cultures were periodically monitored for mycoplasma contamination using Chen's fluorochrome test [11].

Hs294T melanoma cells, kept under a low serum condition (1% FCS) for 24 h, were grown for a period of 48 h in an anaerobic incubator (Concept 400, Jouan, Milan, Italy) flushed with a gas mixture containing 0.3% O_2, 5% CO_2 and 95% N_2. In some experiments, hypoxic tumor cells were grown in media additionated with anti-TNFα-R1 polyclonal antibody (Santa Cruz Biotechnology, Heidelberg, Germany). Tumor cells cultured under hypoxia, in the presence or in the absence of anti-TNFα-R1 polyclonal antibody, were re-oxygenated for 24 h. Parallel cultures of melanoma cells grown in 1% FCS were maintained for the entire period of experiments in normoxic conditions. Normoxic Hs294T melanoma cells were also exposed for 24 h to 20 ng/ml of TNFα (Peprotech House, London, UK) to investigate metalloproteinase release.

Western blotting analysis. Tumor cells were washed with ice cold PBS containing 1 mM Na_4VO_3, and lysed in 100 µl of cell lysis buffer containing: 75 mM Tris–HCl (pH 7.4), 9 M Urea, 0.15 M β-mercaptoethanol. Aliquots of supernatants containing equal amounts of protein (50 µg) in Laemmli buffer were separated on 8% (v/v) SDS–PAGE gel. Fractionated proteins were transferred from the gel to a PVDF nitrocellulose membrane using an electroblotting apparatus (Bio-Rad, Segrate, MI, Italy). Blots were stained with Ponceau red to ensure equal loading and complete transfer of proteins, then they were blocked for 2 h, at room temperature, with Odyssey blocking buffer (Dasit Science, Cornaredo, MI, Italy). Subsequently, the membrane was probed at 4 °C overnight with rabbit anti-HIF-1α polyclonal antibody (Santa Cruz Biotechnology), diluted in a solution of 1:1 Odyssey blocking buffer/T-PBS buffer. The membrane was washed in T-PBS buffer, incubated for 1 hour at room temperature with goat anti-rabbit IgG Alexa Flour 680 antibodies (Invitrogen, Monza, Italy), and then visualized by an Odyssey Infrared Imaging System (LI-COR® Bioscience). Mouse anti-α-tubulin monoclonal antibody (Sigma, Saint Louis, MO, USA) was used to assess equal amount of protein loaded in each lane.

Invasion assay. Invasiveness of Hs294T melanoma cells was determined on Matrigel-coated polycarbonate filters (8 µm pore size) mounted in Boyden's chambers. The coated filters were prepared by using Matrigel suspensions at 250 µg/ml. $2.5x10^4$ Hs294T melanoma cells suspended in 200 µl DMEM 4500 containing 250 µg/ml BSA (DMEM/BSA) were seeded into the upper chamber. 200 µl of DMEM/BSA were added to the lower chamber. After 18–24 h of incubation at 37 °C in a 5% CO_2-humidified atmosphere, non-invading cells were removed mechanically using cotton swabs from the upper side of the filters and the micro-porous membranes were fixed for 1 h in ice-cold methanol. Cells on the lower side of the polycarbonate filters were stained with Diff Quick solution and counted. Migration of Hs294T human melanoma cells was also determined in the presence of 50 µM Ilomastat (Calbiochem, Darmstadt, Germany).

RNA isolation and polymerase chain reaction (PCR). Total RNA extracted from melanoma cells using RNAgents (Total RNA Isolation System, Promega, Madison, WI) was determined spectrophotometrically. Complementary DNA (cDNA) was synthesized from 2 µg of total RNA using 1 µl of ImProm-II™ Reverse Transcriptase (Promega). Aliquots of 2 µl of the cDNA were used for PCR amplification. The specific primers used for the identification of human IL-1β, IL-1βR1, TNFα, TNFαR1, MMP-2, MMP-9, GAPDH were: IL-1α (forward: 5'-TGA ACT GCA CGC TCC GGG ACT C-3', reverse: 5'-TTC TGC TTG AGA GGT GCT GAT G-3', 368 bp product; 34 cycles); IL-1βR1 (forward: 5'-AAG GTG GAG GAT TCA GGA CAT-3', reverse: 5'-AGC CTA TCT TTG ACT CCA CTA-3', 284 bp product; 35 cycles); TNFα (forward: 5'-ACC AGG GAG CCT TTG GTT CTG G-3', reverse: 5'-AAG GCA GCT CCT ACA TTG GGT C-3', 273 bp product; 34 cycles); TNFαR1 (forward: 5'-CAG GAT ACG GAC TGC AGG GAC-3', reverse: 5'-ATT CTC AAT CTG GGG TAG GCA-3', 375 bp product; 35 cycles); MMP-2 (forward: 5'-AGG ATC ATT GGC TAC ACA CC-3', reverse: 5'-AGC TGT CAT AGG ATG TGC CC-3', 534 bp product; 30 cycles); MMP-9 (forward: 5'-CGC AGA CAT CGT CAT CCA GT-3', reverse: 5'-GGA TTG GCC TTG GAA GAT GA-3', 535 bp product; 30 cycles) and GAPDH (forward: 5'-ACC ACA GTC CAT GCC ATC AC-3', reverse: 5'-TCC ACC ACC CTG TTG CTG TA-3', 452 bp product; 27 cycles). All PCR reactions were conducted using 0.05 U/µ of Go-Taq Polymerase (Promega). Amplification was carried out on a Perkin-Elmer Thermal cycler. Eight microliters of each PCR products were visualized after electrophoresis in a 2% agarose gel containing 0.5 µg/ml of ethidium bromide. cDNA products were evaluated on the basis of a standard PCR marker (Promega) and quantified by densitometry. The relative amount of IL-1α, IL-1αR1, TNFα, TNFαR1, MMP-2, MMP-9 mRNA was normalized to GAPDH.

Zymography The gelatinolytic activity released by melanoma cells into their respective growth medium was tested by means of electrophoresis on a 8% SDS-PAGE gel co-polymerized with 0.1% (w/v) type A gelatin (Sigma). Gels were washed for 30 min in 2.5% (v/v) Triton X-100 (Sigma) in order to remove SDS, and then incubated for 24 h at 37 °C in 50 mM Tris-HCl, pH 7.4, containing 200 mM NaCl and 5 mM $CaCl_2$. In order to visualize the zone of lysis, the gels were stained for 60 min at room temperature with 0.1% Coomassie brilliant blue (Merck, Darmstadt Germany), and de-stained for 30 min in a solution of methanol/acetic acid/water (3:1:6; v/v). The gelatinolytic activity was demonstrated as clear bands on a blue background. The gelatinolytic activities of HT1080 human fibrosarcoma cells were used as markers of molecular weight.

Transient transfection. Hs294T melanoma cells were plated into 60 mm cell culture dishes and grown to 30% to 50% confluence. Transfection of tumor cells was performed using Oligofectamine (Invitrogen) with siRNA oligonucleotides targeting HIF-1α (target sequence: 5'-AAA GGA CAA GUC ACC ACA GGA-3') obtained from Quiagen (Valencia, CA) and diluted to a final concentration of 20 nM in DMEM 4500. Ten microliters of Oligofectamine transfection reagent (Invitrogen) was added and the mixture incubated at room temperature for 30 min. Cells were rinsed in DMEM 4500 to remove any residual of FCS before the addition of siRNA complexes and then incubated in serum-free condition for 16 hours at 37°C. Media were then changed into DMEM 4500 supplemented with 10% FCS and cells were incubated for an additional 48 h before using.

Statistical analysis. All experiments were repeated three times. Data are presented as the mean values ± SEM. The statistical significance of the differences was determined using Student's t-test and defined at $p \leqslant 0.05$.

RESULTS AND DISCUSSION

Hypoxia is a characteristic hallmark of solid tumors able to influence expression of a number of genes implicated in survival and tumor spread. [1–3]. We shows here that Hs294T melanoma cells incubated for 48 hours in 0.3% O_2 express HIF-1α protein, measured by Western-blot analysis (Fig. 1, *a*), associated with an increased capacity to penetrate Matrigel-coated filters (Fig. 1, *b*). Hypoxic melanoma cells retains a higher invasiveness, when they were cultivated for 24 h in normoxia, although HIF-1α protein accumulation disappeared (Fig. 1, *c*). Both hypoxic and hypoxic/re-oxygenated tumor cells express a higher MMP-9 activity, evaluated by zymography (Fig. 1, *d*), and consistent with this finding, Ilomastat, an MMP inhibitor, inhibits the enhanced invasiveness

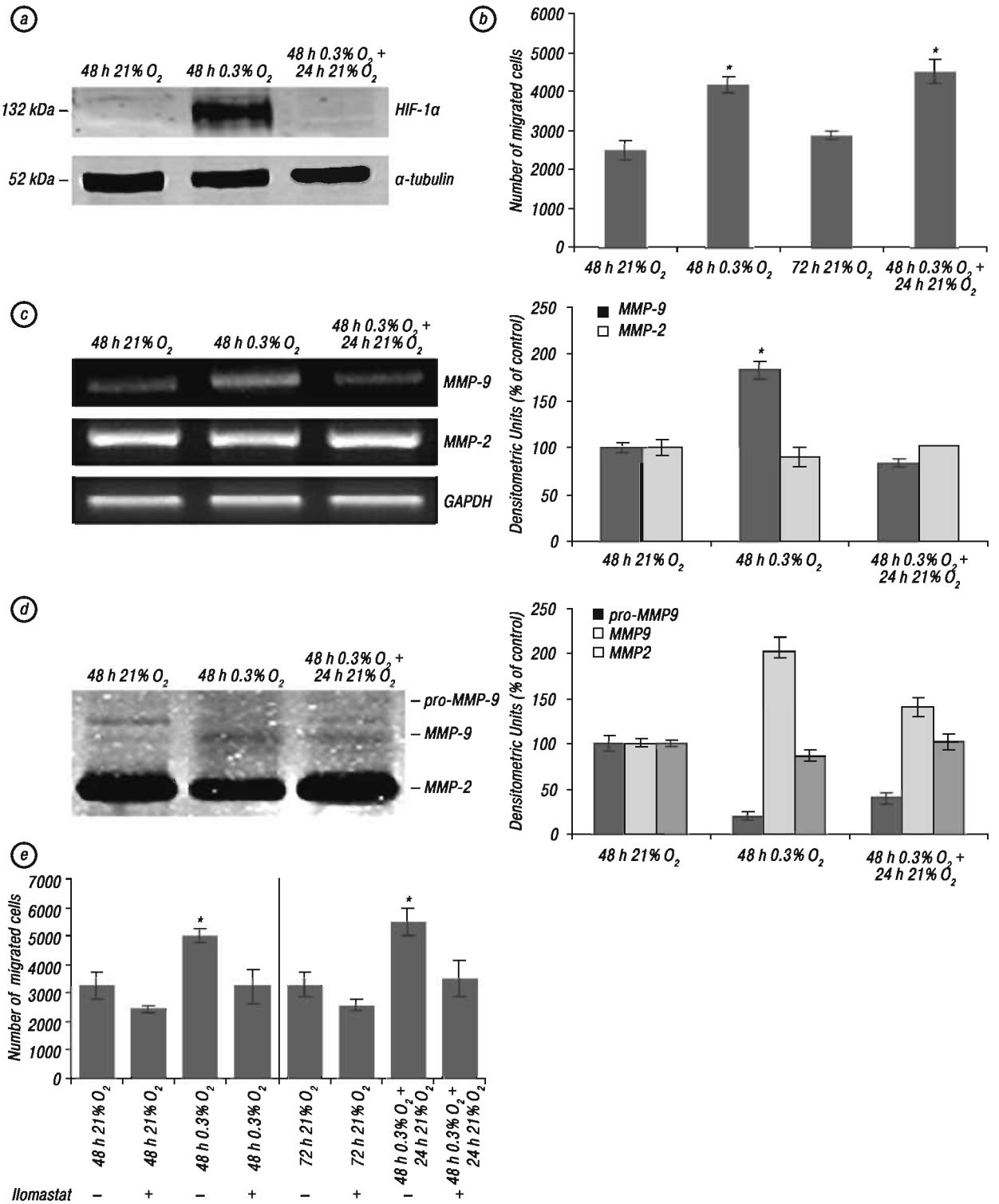

Fig. 1. Expression of HIF-1α protein (*a*) and invasiveness through Matrigel (*b*); RT-PCR analysis of mRNA for MMP-2 and MMP-9 (*c*) and gelatin zymography of tumor cell conditioned media (*d*) with the relative densitometric analysis of experiments; invasiveness through Matrigel in the presence of Ilomastat (*e*). Tumor cells were grown under a normoxic (48–72 h), hypoxic (48 h) or hypoxic (48 h)/re-oxygenation (24 h) condition. * $p < 0.05$

observed in these cells (Fig. 1, *e*). The contribution of MMPs to invasiveness and development of metastases is well ascertained by several studies [12, 13]. Silencing of MMP-9 by RNA interference in human Ewing's sarcoma disclosed that MMP-9 promotes migration of these tumor cells [14]; down-regulation of MMP-9 in prostate carcinoma cells induced a decreased tumor growth accompanied by tumor regression in 50% of animals [15]; downregulation of MMP-9 in glioblastoma cells resulted in a loss to produce intracranial tumors [16].

Considering hypoxia and inflammation are intertwined at the molecular and cellular levels [17, 18] and inflammatory cytokines secreted by tumor-associated

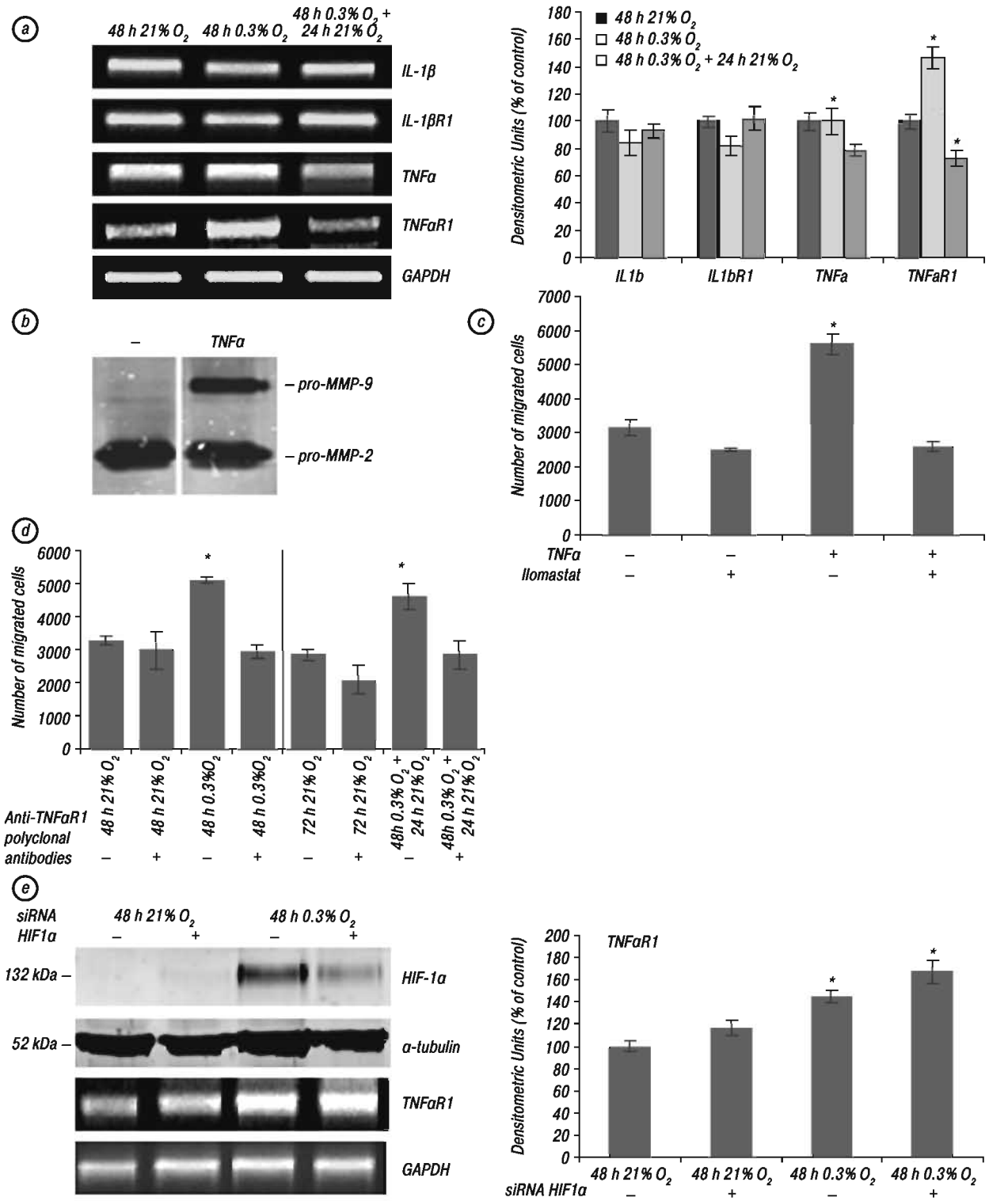

Fig. 2. Representative blot showing changes in IL-1α, IL-1αR1, TNFα, TNFαR1 mRNA and densitometric analysis of experiments (*a*); change in MMP-9 expression (*b*) and invasiveness of melanoma cells stimulated with TNFα in the absence or in the presence of Ilomostat (*c*); change in invasiveness of melanoma cells grown under a hypoxic conditions, in the absence or in the presence of anti-TNFαR1 polyclonal antibodies, and then re-oxygenated (*d*); RT-PCR analysis of mRNA for TNFαR1 in melanoma cells treated with siRNA for HIF1α and grown under normoxic or hypoxic condition with densitometric analysis of TNFαR1 (E). * $p < 0.05$

macrophages and tumor cells themselves promote MMP release [19, 20], we explored whether the enhanced invasiveness of hypoxic melanoma cells might be related to some autocrine activity exerted by inflammatory cytokines on their own receptors. We found that hypoxic Hs294T melanoma cells express an increase level of mRNA for TNFαR1, without any modification of mRNA for IL-1α, IL-1αR1 and TNFα (Fig. 2, *a*). Fig. 2 (*b*, *c*) shows that TNFα stimulates MMP-9 activity and invasiveness of Hs294T melanoma cells, suggesting that a possible increase in TNFα/TNFαR1 interplay is crucial in the enhanced invasiveness acquired by hypoxic melanoma cells. Indeed, addition of polyclonal antibody anti-TNFαR1 to culture media of Hs294T melanoma cells grown in hypoxia and then re-oxygenated abrogates the enhanced invasiveness expressed by both hypoxia and hypoxia/re-oxygenated tumor cells (Fig. 2, *d*). Many cancer cells constitutively express TNFαR1 and secrete TNFα and this appears to increase growth and progression of tumors [21]. Katerinaki et al (2003) reported that TNFα may exert its proinvasive effect on HBL melanoma cells via an integrin-dependent mechanism and a modest upregulation of degradative enzyme activity [22]. The systemic effects, such as fatigue, depression and cognitive impairment of tumor bearing patients are also associated with TNFα [23]. In contrast with tumor cells, it was shown that hypoxia diminishes IL-1αRI and TNFαRI in human endothelial cells, together with Toll-like receptor 4 [24].

We next asked whether HIF-1α could be involved in TNFαR1 up-regulation in hypoxic Hs294T melanoma cells, and we found that siRNA for HIF-1α did not abolish up-regulation of TNFαR1 in hypoxic melanoma cells (Fig. 2, *e*). HIF-1α mediates pleiotropic responses to an hypoxic environment by inducing several genes, although hypoxia activates several others transcription factors, drives genomic instability and alters DNA damage repair pathways [24]. Cooperation between multiple signal pathways to activate a wide array of hypoxia-inducible genes indicates redundant possibilities of cells to adapt to hypoxia.

Altogether, these data suggest that hypoxia promotes the expression of TNFαR1 crucial for melanoma invasiveness, without implicating HIF-1α signaling.

A more comprehensive understanding of endogenous expression of inflammatory cytokine receptors might provide new opportunities for improving strategies against dissemination of melanoma cells.

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