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The biphasic nature of hypoxia-induced directional migration of activated human hepatic stellate cells

Authors

Erica Novo1*, Davide Povero1*, Chiara Busletta1, Claudia Paternostro1, Lorenzo Valfrè di Bonzo1, Stefania Cannito1, Alessandra Compagnone1, Andrea Bandino1, Fabio Marra2, Sebastiano Colombatto1, Ezio David3, Massimo Pinzani2, Maurizio Parola1

*These authors contributed equally to this study.

1 Dip. Medicina e Oncologia Sperimentale—Centro Interuniversitario di Fisiopatologia Epatica, University of Torino, Torino, Italy

2. Dip. Medicina Interna—Centro di Ricerca, Trasferimento e Alta Formazione 'DENOThe', University of Florence, Florence, Italy

3.Pathology Unit, San Giovanni Hospital, Torino, Italy

Corresponding author

E-mail address: maurizio.parola@unito.it

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Abstract

Liver fibrogenesis is sustained by pro-fibrogenic myofibroblast-like cells (MFs), mainly originating from activated hepatic stellate cells (HSC/MFs) or portal (myo)fibroblasts, and is favoured by hypoxia-dependent angiogenesis. Human HSC/MFs were reported to express vascular-endothelial growth factor (VEGF) and VEGF-receptor type 2 and to migrate under hypoxic conditions. This study was designed to investigate early and delayed signalling mechanisms involved in hypoxia-induced migration of human HSC/MFs. Signal transduction pathways and intracellular generation of reactive oxygen species (ROS) were evaluated by integrating morphological, cell, and molecular biology techniques. Non-oriented and oriented migration were evaluated by using wound healing assay and the modified Boyden's chamber assay, respectively. The data indicate that hypoxia-induced migration of HSC/MFs is a biphasic process characterized by the following sequence of events: (a) an early (15 min) and mitochondria-related increased generation of intracellular ROS which (b) was sufficient to switch on activation of ERK1/2 and JNK1/2 that were responsible for the early phase of oriented migration; (c) a delayed and HIF-1α-dependent increase in VEGF expression (facilitated by ROS) and its progressive, time-dependent release in the extracellular medium that (d) was mainly responsible for sustained migration of HSC/MFs. Finally, immunohistochemistry performed on HCV-related fibrotic/cirrhotic livers revealed HIF-2α and haem-oxygenase-1 positivity in hepatocytes and α-SMA-positive MFs, indicating that MFs were likely to be exposed *in vivo* to both hypoxia and oxidative stress. In conclusion, hypoxia-induced migration of HSC/MFs involves an early, mitochondrial-dependent ROS-mediated activation of ERK and JNK, followed by a delayed- and HIF-1α-dependent up-regulation and release of VEGF.

Introduction

Fibrotic progression of chronic liver diseases (CLDs) is sustained by a heterogeneous population of hepatic myofibroblast-like cells (MFs) originating mainly from activation of hepatic stellate cells (HSCs) or portal (myo)fibroblasts 1–5, particularly for chronic biliary diseases 3, as well as circulating, bone marrow-derived stem cells 4, 5 engrafting chronically injured liver. Fully activated, MF-like HSCs (HSC/MFs) and their phenotypic responses are dependent on soluble mediators [mainly platelet-derived growth factor (PDGF), monocyte chemoattractant protein-1 (MCP-1/CCL2), angiotensin II, vascular-endothelial growth factor (VEGF), and angiopoietin-1] 1–6, adipokines 7, reactive oxygen species (ROS), and other factors 8 overexpressed during CLDs. This also includes the ability to migrate towards the site of injury and to align with nascent and established fibrotic septa in response to several polypeptides 8–12, ROS (in either a ligand/receptor-dependent or independent manner) 11 or, as previously shown, hypoxia 10.

Hepatic angiogenesis is a dynamic, hypoxiastimulated, and growth factor-dependent process, which is observed during CLDs and has been proposed to favour fibrogenic progression. Overexpression of VEGF, a pivotal pro-angiogenic cytokine, is strictly associated with hypoxic areas and mostly limited to hepatocytes and HSC/MFs (reviewed in refs 13–16). Moreover, HSC/MFs have been proposed to actively contribute to the cross-talk between angiogenesis and liver fibrogenesis, through their ability to express pro-angiogenic cytokines during CLDs. Along these lines, we previously reported 10 that human HSC/MFs exposed to hypoxia migrate in a wound healing assay in relation to hypoxia-dependent up-regulation of VEGF-A, Ang-1, and related receptors (VEGFR-2 and Tie2). The overall scenario indicates that VEGF and Ang-1 contribute to fibrogenesis acting as hypoxia-inducible, autocrine, and paracrine factors able to recruit MFs.

In the present study, we identify and characterize a previously unrecognized biphasic nature of this process, further investigating the mechanisms involved in hypoxia-induced migration of human HSC/MFs. We suggest the existence of an early phase of hypoxia-induced migration that is switched on by ROS released by mitochondria and is related to redox-dependent activation of Ras/ERK and JNK. In contrast, a delayed and sustained phase of migration mainly depends on HIF-1α-mediated, ROS-stabilized up-regulation of VEGF expression, resulting in the subsequent chemotactic action of extracellularly released VEGF.

Materials and methods

Materials

Human recombinant growth factors and cytokines were from PeproTech Inc (Rocky Hill, NJ, USA). Monoclonal and polyclonal antibodies against phosphorylated and unphosphorylated Erk1/2, against HIF-1α, and VEGF were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); the polyclonal antibodies against phosphorylated and unphosphorylated JNK1/2 were from Cell Signaling Technology (Beverly, MA, USA). SP600125 and PD98095 were from Calbiochem (La Jolla, CA, USA). The enhanced chemiluminescence reagents and nitrocellulose membranes (Hybond-C extra) were from Amersham Pharmacia Biotech (Milano, Italy). All other reagents were from Sigma Aldrich Spa (Milan, Italy).

Isolation and culture of hepatic stellate cells (HSCs)

Human HSCs were isolated and characterized 17 from surgical wedge sections of at least three different human livers not suitable for transplantation after obtaining the approval of the Human

Research Review Committee (University of Florence). HSCs were cultured as previously described 10–12, used between passages 4 and 8 (fully activated HSC/MFs), plated to obtain the desired sub-confluence level, and then left for 24 h in serum-free Iscove's medium to have cells at the lowest level of spontaneous proliferation. In experiments designed to evaluate the role of hypoxia, as previously detailed 7, 10, serum-deprived and sub-confluent hHSC/MFs (60–70%) were incubated in strictly controlled hypoxic conditions (3% O_2).

Cell migration and chemotaxis

Migration of human HSC/MFs was evaluated by performing the wound healing assay (20 h of incubation) or the modified Boyden's chamber assay (6 h of incubation), as previously described 10–12.

Molecular biology procedures

Total cell extracts were subjected to SDS-PAGE on 10% or 7.5% acrylamide gels. The blots were incubated with the desired primary antibodies and then with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in Tris-buffered saline–Tween containing 2% (w/v) non-fat dry milk 10, 11, 18 and developed with the enhanced chemiluminescence reagents according to the manufacturer's instructions.

The target siRNA sequence for down-regulation of human JNK isoforms (5-GAAAGAATGTCCTACCTTCT-3) is found in both *JNK1* mRNA (nucleotides 393–412) and *JNK2* mRNA (nucleotides 425–444) 19. The target siRNA sequences for down-regulation of human HIF-1α and HIF-2α are HIF-1α: 5-AGGAAGAACTATGAACATAAA-3 and HIF-2α: 5′-CCCGGATAGACTTATTGCCAA-3′.

Effective silencing of either human JNK isoforms in HSC/MFs 11 or human HIF-1α in other human cells 20 using these siRNAs was recently reported. JNK1/2, HIF-1α, and HIF-2α protein levels were analysed by western blot analysis 96 and 120 h after transfection, respectively.

siRNAs and related non-silencing controls were synthesized by Qiagen-Xeragon (Germantown, MD, USA). For transfection, the Amaxa nucleofection technology (Amaxa, Koln, Germany) was employed 21.

Detection of intracellular levels of ROS

Intracellular levels of ROS were detected by means of the semi-quantitative 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence technique, as previously detailed 20, in cells exposed to the desired stimulus for 15 min or to 50 μM hydrogen peroxide (H_2O_2, positive control).

Morphological analysis

Indirect immunofluorescence was performed on hHSC/MFs exposed to controlled hypoxic conditions and on hHSC/MFs pretreated with DPI, rotenone, SP600125, PD98095, and then exposed to hypoxia, as previously described 10, 11, 20. Final dilution of primary antibodies was 1 : 100 (HIF-1α) and immunopositivity was revealed by the appropriate secondary Cy3-conjugated antibody (1 : 1000 dilution; Amersham Pharmacia Biotech, Milano, Italy). Nuclei were stained using 4,6-diamino-2-phenylindole (DAPI).

Immunohistochemistry was performed on paraffin liver sections from patients with hepatitis C virus (HCV)-related liver cirrhosis (METAVIR F4). The use of this material conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the University of Florence Human Research Review Committee. Sections (2 μm thick) were incubated with specific antibodies raised against HIF-2α, HO-1 or α-SMA (final dilutions 1 : 100 and 1 : 1000, respectively). Briefly, after microwave antigen retrieval, primary antibodies were labelled by using EnVision, HRP-labelled System (DAKO) antibodies directed against rabbit antigen and visualized by 3′-diaminobenzidine substrate. Negative controls were performed by replacing the respective primary antibodies by isotype- and concentration-matched irrelevant antibody.

Statistical analysis

Data in the bar graphs represent means ± SEM and were obtained from average data of at least three independent experiments. Luminograms and morphological images are representative of at least three experiments with similar results. Statistical analysis was performed by Student's *t*-test or ANOVA for analysis of variance when appropriate ($p < 0.05$ was considered significant).

Results

Hypoxia-induced early migration of human HSC/MFs is independent of HIF-1α and VEGF

We previously reported that exposure of human HSC/MFs to controlled conditions of moderate hypoxia results in significant stimulation of non-oriented migration (Supporting information, Supplementary Figure 1A). Moreover, conditioned medium collected from cells exposed to hypoxia for 16 or 24 h induces significant stimulation of both chemokinesis and chemotaxis, an effect attributed to hypoxia-dependent up-regulation of VEGF expression and its delayed release in the extracellular medium 10 (Supporting information, Supplementary Figures 1A–1C). To further investigate the mechanisms involved in hypoxia-induced migration of HSC/MFs, we first analysed the effects of hypoxia alone on early (ie 6 h) oriented migration of hHSC/MFs, performing a Boyden chamber assay. Hypoxia was sufficient to induce a significant increase in cell migration (Supporting information, Supplementary Figure 1B). Moreover, SU1498, a VEGF-R2 pharmacological inhibitor preventing hypoxia-induced and VEGF-dependent migration in the WHA 10 (Supporting information, Supplementary Figure 1A), failed to affect early migration (Supporting information, Supplementary Figure 1B).

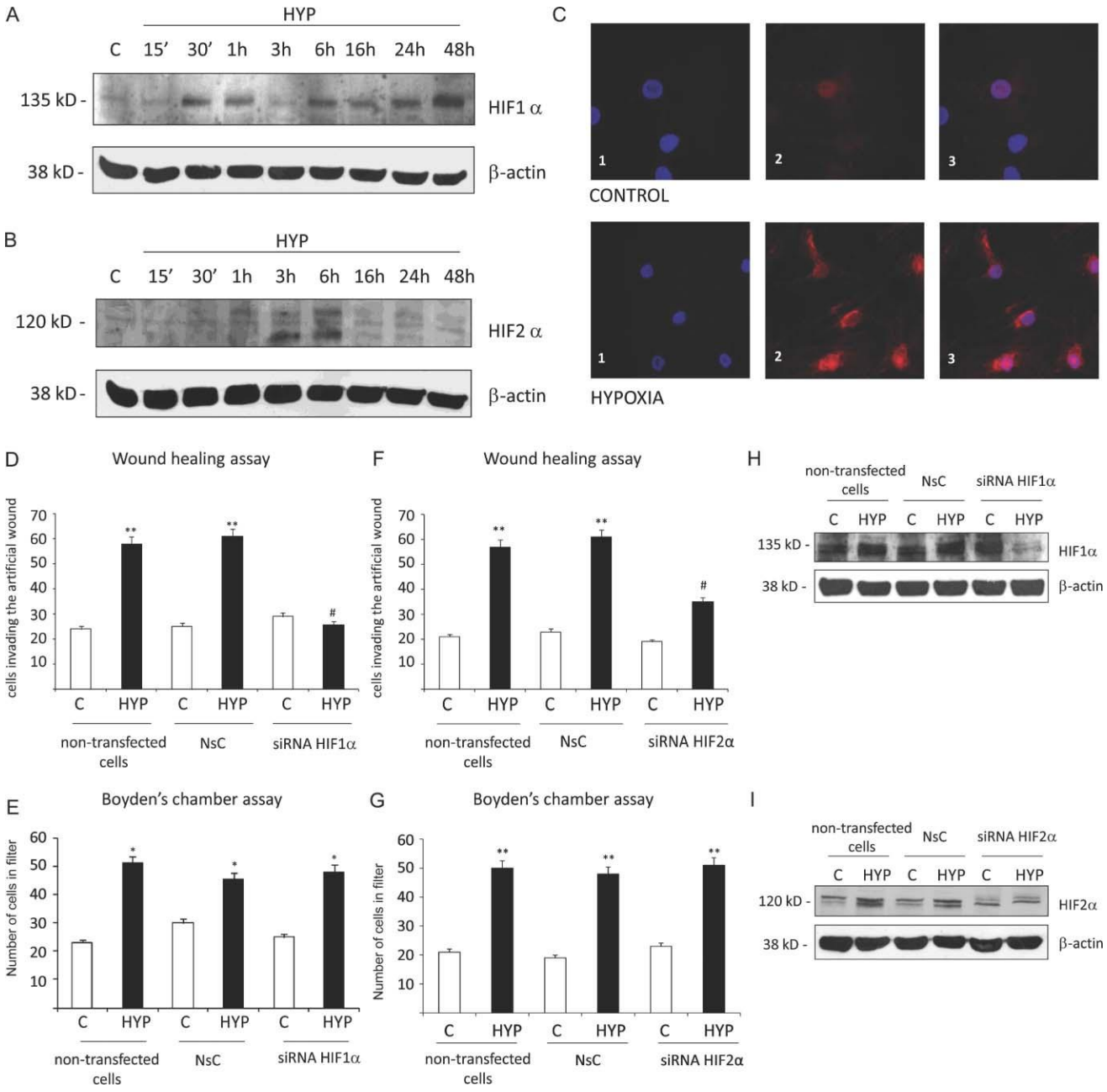

Figure 1.

Hypoxia-inducible factors and hHSC/MF migration. Confluent and 24 h starved hHSC/MFs were exposed to hypoxic conditions (3% O_2) for the indicated time points. HIF-1α (135 kD) (A) and HIF-2α (120 kD) (B) were detected by western blot analysis on total cell lysates. Sample loading was evaluated by re-blotting the same membranes for β-actin. Levels of HIF-1α were also evaluated by means of indirect immunofluorescence in hHSC/MFs maintained for 1 h in normoxic conditions (control) or exposed to hypoxia (C). For any condition, three images are offered of the same field representing nuclear DAPI fluorescence (C, panel 1), immunopositivity for HIF-1α (C, panel 2), and the result of their electronic merging (C, panel 3). hHSC/MFs were efficiently silenced for HIF-1α as detailed in the Materials and methods section. Migration of hHSC/MFs in both the WHA (D) and Boyden's chamber (E) was assessed in cells not transfected, cells transfected with a non-silencing control siRNA (NsC), or cells transfected with HIF-1α siRNA; cells were next exposed either to normoxic conditions (control cells) or to hypoxia. Similarly, hHSC/MFs were then

efficiently silenced for HIF-2α and both non-oriented migration (WHA) (F) and chemotaxis of hHSC/MFs in Boyden's chamber (G) were assessed in cells not transfected, cells transfected with a non-silencing control siRNA (NsC) or cells transfected with HIF-2α siRNA; cells were next exposed either to normoxic conditions (control cells) or to hypoxia. Western blot analysis confirmed the efficiency of siRNA-mediated silencing of HIF-1α (H) and HIF-2α (I) in the previously indicated experimental conditions. Data in the bar graphs represent mean ± SEM ($n = 4$, in triplicate) and are expressed as the number of cells migrated in the artificial lesion or in the filter of Boyden's chambers. *$p < 0.05$ and **$p < 0.01$ versus control values. $^{\#}p < 0.01$ versus values in cells exposed to hypoxia and not treated with HIF-1α siRNA

We next investigated the involvement of HIF-1α (known to form HIF-1 heterodimers able to up-regulate VEGF expression) in our experimental conditions. As expected, under hypoxic conditions HIF-1α was recruited/stabilized at an early time point (within 30–60 min) and its activation lasted until 24–48 h (Figure 1A). In addition, hypoxia led to a similar recruitment/stabilization of HIF-2α, although maximal protein levels were slightly postponed (Figure 1B). The involvement of HIF-1α was confirmed by its early (1 h) nuclear translocation under hypoxia (see Figure 1C). However, efficient silencing of HIF-1α, which resulted in very significant inhibition of late migration (WHA at 20 h, Figure 1D), was essentially ineffective on early hypoxia-induced oriented migration in the Boyden's chamber assay (Figure 1E). Concerning early migration, homologous results were obtained by silencing HIF-2α, a procedure that was again effective on the WHA but ineffective on early hypoxia-induced oriented migration in the Boyden's chamber assay (Figures 1F and 1G). The efficiency of siRNA-mediated silencing for HIF-1α and HIF-2α is presented in Figures 1H and 1I, respectively.

Early hypoxia-induced oriented migration depends on mitochondrial ROS generation and redox- dependent activation of ERK1/2 and JNK1/2 signalling

In a previous study, we described a cause–effect relationship between early generation of ROS, activation of ERK and JNK signalling, and migration of human HSC/MFs under normoxic conditions 11. It is well known that ROS are significantly released in the cytoplasm by mitochondria under hypoxic conditions 20, 22, 23. In HSC/MFs cultured under hypoxia, intracellular ROS generation was detected at an early time point (15 min–1 h) by the DCFH-DA semi-quantitative technique (Figure 2A). Moreover, pretreatment with two inhibitors of the mitochondrial electron transport chain, such as rotenone (ROT) and diphenyl-phenylene iodonium (DPI), resulted not only in significant inhibition of intracellular ROS generation (Figure 2A), but also in very significant inhibition of hypoxia-induced early migration (Figure 2B). DPI (1 μM) and ROT (2.5 μM) were employed at concentrations that were unable to induce either necrotic or apoptotic cell death; irreversible cell injury, as monitored in terms of LDH release (Supporting information, Supplementary Figure 2A) or DAPI staining (Supporting information, Supplementary Figure 2B), was observed for concentrations of DPI and ROT higher than 100 and 25 μM, respectively.

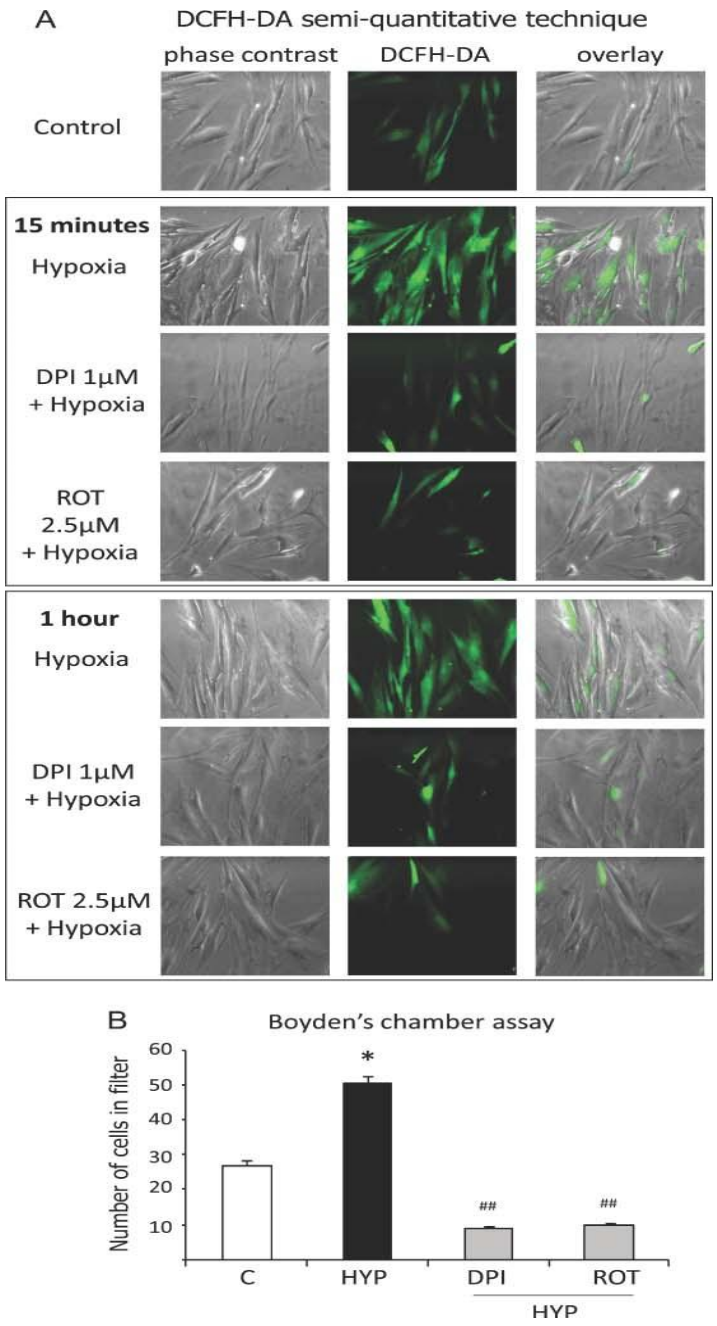

Figure 2.

Hypoxia-related ROS and early migration of hHSC/MFs. Generation of intracellular ROS (green fluorescence) was detected by the semi-quantitative technique based on the use of DCFH-DA (A) at 15 min and 1 h in cells maintained in normoxic conditions (control) and cells exposed to hypoxia or pretreated with diphenyl-phenylen iodonium (DPI, 1 μM) or rotenone (2.5 μM) before exposure to hypoxia. For any condition, three images are offered of the same field, representing phase contrast image (left column), DCFH-DA-positive fluorescence (middle column), and their digital overlay (right column). Pretreatment of cells with DPI or rotenone before exposure to hypoxia inhibited hHSC/MF chemotaxis (B). $*p < 0.05$ versus control values. $^{\#\#}p < 0.1$ versus values in cells exposed to hypoxia

Under hypoxic conditions, we next detected early (15–30 min) as well as sustained (16 and 24 h) activation/phosphorylation of ERK1/2 and JNK1/2 isoforms, with a prevalent involvement of 46 kD JNK1/2 (Figure 3A). The relevance of ERK and JNK signalling for hypoxia-induced oriented

migration was shown by inhibition of the early migration of HSC/MFs in the presence of pharmacological inhibitors of ERK1/2 (PD98095) or JNK1/2 (SP600125) activation (Figure 3B), which were not significantly affecting basal migration under normoxic conditions.

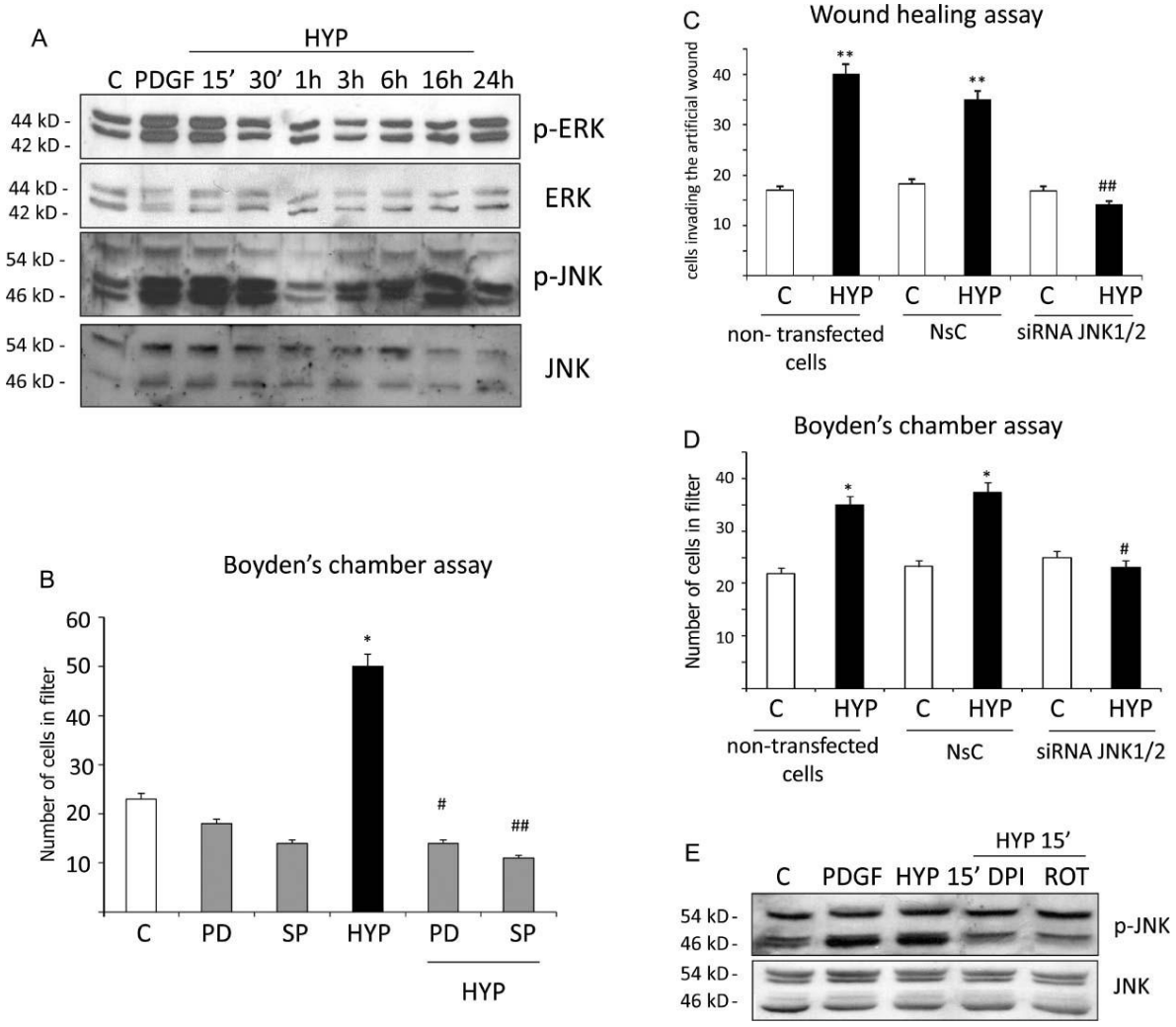

Figure 3.

ERK1/2 and JNK1/2 signalling in hypoxia-induced migration. Confluent and 24 h-starved hHSC/MFs were incubated for 15 min under normoxic conditions in the absence (control) or in the presence of PDGF-BB (10 ng/ml, positive control) or exposed to hypoxia for the indicated time points. Levels of phosphorylated and unphosphorylated ERK1/2 (p44 and p42) and JNK1/2 (p46 and p54) were detected by western blot analysis on total cell lysates (A). Boyden's chamber assay (B) was performed on hHSC/MFs maintained in normoxic conditions (control), exposed to hypoxia, or pretreated, under normoxia or before exposure to hypoxia, with the pharmacological inhibitors of MEK1 (PD98095, 30 µM) or JNK1/2 (SP600125, 20 µM), respectively. Data in the bar graphs represent mean ± SEM (n = 4, in triplicate) and are expressed as the number of cells migrated in the artificial lesion or in the filter of Boyden's chambers. *p < 0.05 and **p < 0.01 versus control values. $^{\#}p$ < 0.01 and $^{\#\#}p$ < 0.05 versus values in cells exposed to hypoxia alone. hHSC/MFs were silenced for JNK1/2 as described in the Materials and methods section (C, D). Migration in the WHA (C) and in Boyden's chamber (D) was assessed in cells that were not transfected, cells transfected with a non-silencing control siRNA (NsC), or cells transfected with the desired siRNA. Cells were then either maintained in normoxic conditions (control cells) or exposed to hypoxia for the indicated time. Data in the bar graphs represent mean ± SEM (n = 4, in triplicate) and are

expressed as the number of cells migrated in the artificial lesion or in the filter of Boyden's chambers. *$p < 0.05$ and **$p < 0.01$ versus control values. $^{\#}p < 0.01$ and $^{\#\#}p < 0.1$ versus values in cells exposed to hypoxia and not treated with JNK1/2 siRNA. Pretreatment of cells with the inhibitors of mitochondrial electron transfer (complex I) diphenyl-phenylen iodonium (DPI, 1 μM) or rotenone (2.5 μM) before exposure to hypoxia completely abolished hypoxia-induced activation/phosphorylation of 46 kD JNK1/2 isoforms (E)

According to the recently reported relevance of JNK1/2 for HSC/MF migration 11 and liver fibrogenesis 24, 25, we next employed gene silencing by siRNA to down-regulate JNK1/2 (particularly the 46 kD isoforms) 11. Effective silencing of JNK1/2 isoforms resulted in very significant inhibition of the migration of HSC/MFs exposed to hypoxia in both WHA and Boyden's chamber assay (Figures 3C and 3D). Moreover, pretreatment of HSC/MFs with either DPI or ROT almost completely abolished hypoxia-induced activation of 46 kD JNK1/2 isoforms (Figure 3E).

Since JNK1/2 silencing was effective in inhibiting both early and delayed migration, we decided to evaluate whether ROS may contribute to HIF-1α recruitment/stabilization. Pretreatment of HSC/MFs with either ROT or DPI resulted in a decrease of HIF-1α recruitment/stabilization and nuclear translocation (Supporting information, Supplementary Figure 3A). In addition, the simple intracellular rise of ROS levels, as obtained in normoxic conditions following treatment with either menadione, DMNQ or the hypoxanthine/xanthine oxidase system 11, 12, was sufficient to increase HIF-1α recruitment/stabilization (Supporting information, Supplementary Figure 3B).

Finally, to provide an *in vivo* counterpart to the data obtained in cell culture, we performed immunohistochemistry for HIF-2α, the redox-dependent factor HO-1, and α-SMA on serial liver sections obtained from patients with HCV-related cirrhosis. In fact, these markers are related to hypoxia, oxidative stress, and MFs, respectively (Figures 4A–4D). Positive nuclear staining for HIF-2α or cytoplasmic staining for HO-1 was detected in hepatocytes of regenerative nodules (particularly in hepatocytes close to fibrotic septa) as well as in cells of fibrotic septa, including α-SMA-positive MFs. In particular, as far as MF-like cells are concerned, image analysis suggests that this scenario is mainly evident at the border of mature and larger fibrotic septa (and less evident in the core or central structures of large septa; see Figures 4A and 4B) as well as in MFs of developing septa (Figures 4C and 4D). Moreover, positive staining for the antigens of interest (HIF-2α, HO-1, and α-SMA) was negligible in non-cirrhotic normal human liver (see Supporting information, Supplementary Figure 4).

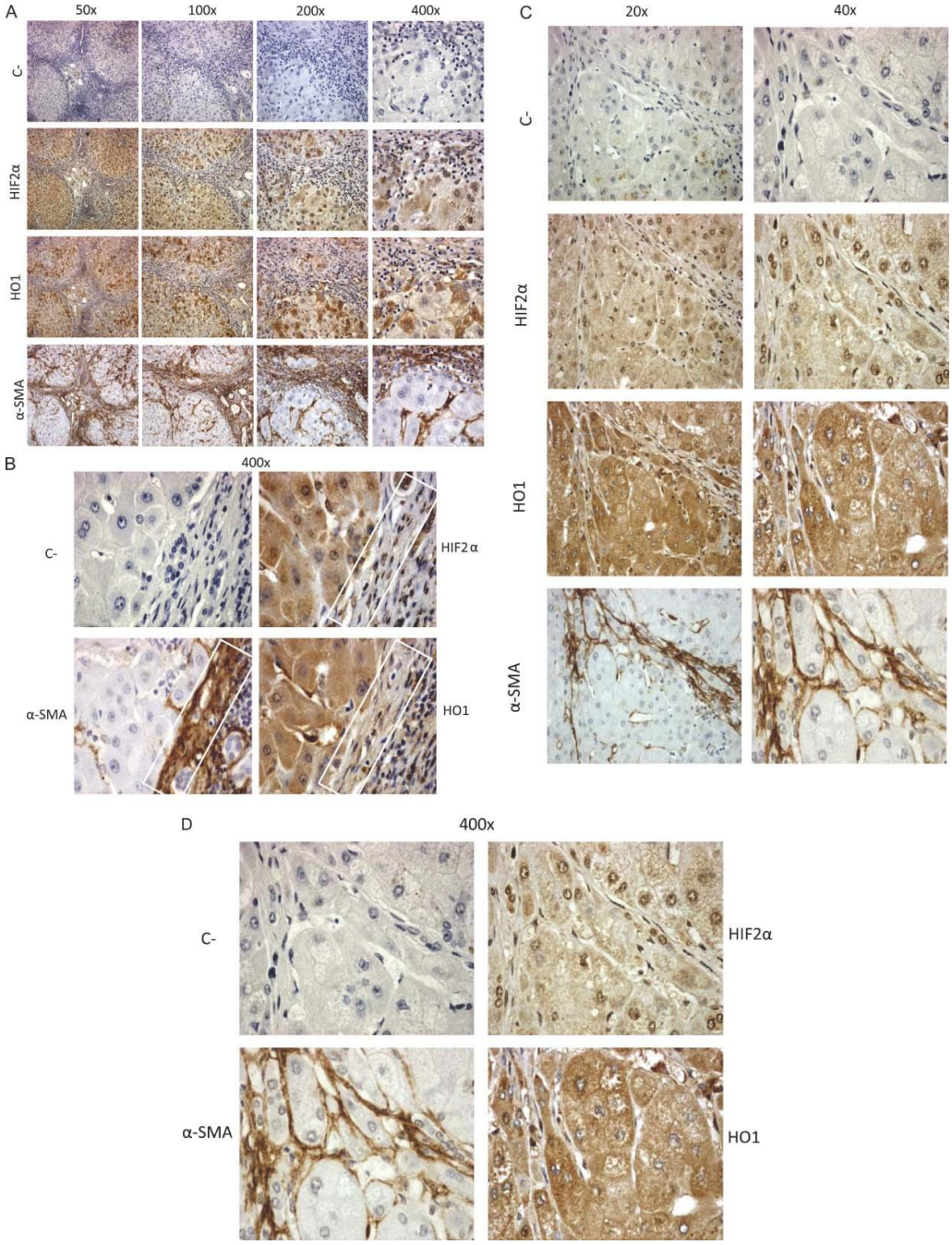

Figure 4.

Hypoxia, haem oxygenase-1, and α-SMA in human cirrhosis. Immunohistochemistry was performed on paraffin serial sections from patients with hepatitis C virus (HCV)-related liver cirrhosis (Metavir F4) using (A–D) antibodies against α-SMA, HIF-2α or haem oxygenase-1 (HO-1). Panels A and B are related to large fibrotic septa, whereas panels C and D are related to thin and developing septa. Panels indicated as C- represent the negative control. Original magnification as indicated

These data suggest that MFs in cirrhotic human livers, particularly those of developing septa and those at the border of larger and more mature septa, are likely to be exposed to both hypoxia and oxidative stress.

Discussion

Migration of HSC/MFs and hepatic MFs of different origins represents a distinctive pro-fibrogenic feature leading MFs to align with inflammatory cells along fibrotic septa. This occurs in response to either chemoattractant polypeptides or other mediators generated during CLDs 1–6, including ROS 7, 11. Hypoxic conditions are common in either clinical or experimental CLDs, and stimulate angiogenesis that favours fibrogenic progression of the disease 13–16. In a previous study, we provided evidence 10 that human HSC/MFs exposed to hypoxia migrate in a wound healing assay following hypoxia-dependent up-regulation of VEGF expression and release, with VEGF envisaged as an autocrine/paracrine factor able to recruit MFs.

The present study was designed to investigate the molecular mechanisms underlying the up-regulation of VEGF in HSC/MFs in conditions of hypoxia. The data provide evidence for the first time that hypoxia-induced migration of human HSC/MFs occurs as a biphasic process according to the following sequence: (a) an early phase of hypoxia-induced migration triggered by ROS released within minutes by mitochondria that are responsible for pro-migratory, redox-dependent activation of Ras/ERK and JNK signalling pathways; and (b) a delayed and sustained phase of migration, mainly dependent on HIF-1α-mediated, ROS-stabilized up-regulation of VEGF expression followed by its extracellular release and paracrine chemotactic action.

The dissection between an early and a late phase of hypoxia-induced migration is strongly supported by the following evidence: (a) a hypoxia-dependent significant increase in HIF-1α (as well as of HIF-2α) recruitment/stabilization is rapidly detected in HSC/MFs (within 30–60 min) and persists throughout the entire experimental protocol; (b) however, VEGF, which stimulates both delayed non-oriented migration and chemotaxis of human HSC/MFs 10, 11 (this study), is released in the extracellular medium by hypoxic HSC/MFs only after 16 h from exposure to hypoxia 10; (c) pretreatment of cells with SU1498, a pharmacological inhibitor of the tyrosine-kinase receptor subunit of VEGFR-2, inhibits non-oriented delayed migration in the WHA but fails to affect early hypoxia-induced oriented migration; (d) delayed paracrine action of VEGF is also suggested by the reported late (ie 16–24 h) increase in phosphorylation of ERK1/2 and JNK1/2; (e) finally, silencing of HIF-1α (and then of VEGF) resulted in significant prevention of hypoxia-related delayed migration of HSC/MFs in the WHA but was ineffective on early oriented migration. These data indicate that hypoxia-dependent involvement of HIF-1α and VEGF, previously hypothesized for human HSC/MFs 11 and described for other cells 20, 26, is then effective in sustaining the delayed phase of HSC/MF migration under hypoxic conditions.

The analysis of early signalling events following hypoxia revealed early activation of ERK1/2 and JNK1/2, similar to the pro-migratory and redox-sensitive series of events that we recently described for HSC/MFs exposed to either chemoattractant polypeptides, which generate intracellular ROS through the involvement of NADPH-oxidase, or molecules increasing ROS levels in a receptor-independent manner 11. Indeed, according to current literature 20, 22, 23, exposure of human HSC/MFs to hypoxia was followed within minutes by a significant rise in intracellular ROS mainly released by mitochondria, as unequivocally shown by employing the DCFH-DA technique in the presence of the inhibitors of the mitochondrial electron transport chain, rotenone and DPI. Both inhibitors act as blockers of mitochondrial complex I, preventing ROS release at the level of complex III, and they almost completely abolished not only the rise in intracellular ROS, but also early HSC/MF migration in Boyden's chamber.

In this connection, the relationships between hypoxia and ROS generation outlined in HSC/MFs have been corroborated by an '*in vivo*' counterpart with IHC analysis performed on serial sections obtained from HCV cirrhotic patients. We observed positive nuclear staining for HIF-2α (proof of

exposure to hypoxic conditions) or cytoplasmic staining for HO-1 (a redox-dependent target gene) not only in hepatocytes of regenerative nodules, but also in several cells of fibrotic septa having morphology of α-SMA-positive MFs. In particular, similarly to what has already been described for the expression of pro-angiogenic cytokines (VEGF, angiopoietin 1) and related receptors (VEGF-R2, Tie-2) 10, positive staining for both HIF-2α and HO1 is mainly evident in MF-like cells in developing septa and at the border of more mature and larger fibrotic septa.

Once established a direct relationship between hypoxia-dependent mitochondrial release of ROS and early migration, our data identify at least two distinct roles for the rise of ROS intracellular levels. The first one is mechanistically related to the switch of early hypoxia-dependent migration and is likely to operate through the early redox-dependent phosphorylation/activation of JNK1/2 isoforms. This concept is supported by the following evidence: (a) JNK1/2 isoforms, particularly 46 kD isoforms, are phosphorylated at an early time point under hypoxic conditions, kinetics that is fully compatible with that of mitochondrial ROS release; (b) early oriented migration of HSC/MFs in Boyden's chambers is abolished by all experimental conditions that block JNK1/2 isoforms or mitochondrial ROS release, including the use of the JNK pharmacological inhibitor SP600125, the use of specific JNK1/2 silencing, or pretreatment with the inhibitors of the mitochondrial electron transport chain. Along these lines, the direct link between the rise in intracellular levels of ROS and redox-related activation of both ERK1/2 and JNK1/2 is well established in the setting of redox signalling. Increased kinase activity may result either from direct activation (ie through redox-mediated rearrangement of the 3D structure in monomeric isoforms or heterodimeric complexes) or from inhibition of phosphatases responsible for the negative feed-back control of activated signalling pathways (such as protein tyrosine phosphatases or JNK-specific phosphatases) 8. Moreover, it is well known that activation of MAPKs, in particular JNK isoforms, has an unequivocal and mechanistic relationship with the migration of human cells (reviewed in ref 27).

A second role for the hypoxia-related rise in intracellular ROS levels is related to the contribution to increased HIF-1α stabilization, as already proposed for other cell types 28, 29. This event has been mainly ascribed to a ROS-related inhibition of prolyl-hydroxylases, the enzymes that in normoxic conditions hydroxylate HIF-1α, allowing its binding to VHL protein and subsequent ubiquitination and proteasomal degradation 28, 29. However, ROS-related HIF-1α stabilization and up-regulation of target genes may also rely on redox-dependent kinase activation, where HIF-1α is a downstream signalling target for either MAPKs- 20, 28, 29 or AMPK-mediated 30 phosphorylation. Indeed, data in the present study and those obtained in a previous one 20 suggest that both types of ROS-dependent events may indeed contribute to HIF-1α stabilization under hypoxia.

All the results obtained in the present and in a previous 11 study, including those indicating that JNK1/2 silencing can prevent hypoxia-induced migration of HSC/MFs in both a WHA and Boyden's chamber, are consistent with the view that a simple increase in the intracellular levels of ROS, whatever the specific event involved (ie mitochondrial release, ligand-receptor-mediated activation of plasma membrane NADPH-oxidase, entry of hydrogen peroxide from the extracellular necro-inflammatory environment, etc), can be sufficient to induce migration in motile cells.

The overall scenario emerging from the present study further underlines the critical relevance of hypoxia-related events, oxidative stress, and JNK for HSC activation and fibrogenesis progression, with JNK representing a putative therapeutic target for antioxidants and/or small molecules such as protein kinase inhibitors.

Acknowledgements

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Abbreviations
AMPKadenosine monophosphate-activated protein-kinase CLDs chronic liver diseases DAPI 4,6-diamino-2-phenylindole DCFH-DA 2′,7′-dichlorodihydrofluorescein diacetate DMNQ 2,3-dimethoxy-1,4-naphthoquinone DPI diphenyl-phenylene iodonium ERK1/2 extracellular regulated kinase 1/2 H_2O_2 hydrogen peroxide HCV hepatitis C virus HIF-1α hypoxia-inducible factor 1α HIF-2α hypoxia-inducible factor 2α HNE 4-hydroxynonenal HO-1 haem oxygenase 1 HSCs hepatic stellate cells HSC/MFs activated, myofibroblast-like hepatic stellate cells JNK1/2 c-Jun N-terminal kinase isoforms 1/2 MCP-1 or CCL2 monocyte chemoattractant protein-1MENmenadioneMFsmyofibroblast-like cellsNsCnon-silencing siRNAPDGFplatelet-derived growth factorROSreactive oxygen speciesROTrotenoneα-SMAsmooth muscle actin alphaVEGFvascular endothelial growth factorVEGFR-2VEGF receptor type 2X/XOxanthine-xanthine oxidase

Author contribution statement

EN and DP conceived and carried out experiments as well as analysis of data. CP, CB, LVB, SC, AC, and AB carried out experiments and were involved in data analysis. ED was involved in the analysis of liver specimens and discussed data. FM, SC, MPi, and MPa conceived experiments and discussed data. All the authors were involved in writing the manuscript.

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