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Interleukin-1β regulates the migratory potential of MDAMB231 breast cancer cells through the hypoxia-inducible factor-1α ☆

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ABSTRACT

The casual relationship between inflammation and tumour progression has been widely accepted and the etiology of breast cancer has been associated with inflammatory processes. Interleukin (IL)-1β, besides its central role in inflammation, has also been recognised as a powerful player in tumour progression, angiogenesis and invasiveness. Recently, there has been considerable interest in understanding the non-hypoxic upregulation of the hypoxia-inducible factor (HIF)-1α by IL-1 in neoplastic cells since aberrant expression of HIF-1α correlates with tumour progression. Here, using the highly invasive human breast cancer cell line MDAMB231, we studied the effect of IL-1β on tumour cell migration along with HIF-1α accumulation. We observed that non-hypoxic induction of HIF-1α by IL-1β in MDAMB231 was associated with increased cell migration, paralleled by upregulation of p38 MAPK phosphorylation and CXCL8/CXCR1 expression. Inhibition of HIF-1α by siRNA resulted in a significant reduction of CXCR1 expression and IL-1β-induced cell migration in MDAMB231 cells, thus confirming a role of HIF-1α in the non-hypoxic-IL-1β-dependent induction of migratory potentials. Our observation that IL-1 induces HIF-1α accumulation in MDAMB231 cells was confirmed in tumour cells growing *in vivo* using an experimental approach, mimicking the endogenous release of IL-1 in mice bearing MDAMB231 xenografts. Our *in vivo* data, along with the fact that inhibition of HIF-1α resulted in the decrease of IL-1β-promoted cell migration, further support the link between inflammation and cancer. The overall results may have important implications in those therapeutic approaches aimed to inhibit IL-1-mediated activities in tumour cells, specifically in breast cancer.

1. Introduction

Growing evidence indicates that inflammation may contribute to tumour progression, as pro-inflammatory cytokines and chemokines influence a variety of cell functions, including survival, proliferation and motility of tumour and stromal cells.[1] Indeed, the tumour microenvironment is characterised by the presence of a specific cytokine network, which is particularly rich in inflammatory cytokines, growth factors and chemokines, secreted by malignant and/or stromal cells.[1]

Interleukin (IL)-1β is the prototypical pro-inflammatory cytokine, characterised by multifunctional properties and produced mainly by monocytes/macrophages.[2] Host leucocytes are present in the tumour microenvironment, and such

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presence coincides with the expression of IL-1β in most tumours, including epithelial cancers.[3] In various experimental models, IL-1 mediates tumour invasiveness and metastasis, as well as angiogenesis.[4–6] The secretion of IL-1β into the tumour milieu induces the production of angiogenic factors from tumour and stromal cells and thus promotes tumour growth through neovascularisation.[7] In particular, IL-1β is a potent stimulus for CXCL8 RNA stabilisation in breast cancer cells.[8] The chemokine CXCL8 is secreted by a variety of normal and tumoural cells and acts through its binding to two cell surface CXC chemokine receptor 1 (CXCR1, CXCL8R1 or type A) and CXC chemokine receptor 2 (CXCR2, CXCL8R2 or type B). While CXCR2 binds multiple CXC chemokines, CXCR1 is more specific and its transcription is mediated by the hypoxia-inducible factor (HIF)-1α.[9] The interaction between CXCL8 and its receptors plays an integral role in promoting chemotaxis of tumour cells, including melanoma. Very recently, CXCL8 has been identified in breast cancer stem cells indicating its relevance in breast cancer progression.[10]

Many cytokines and chemokines are inducible by hypoxia, which is a common feature of the tumour microenvironment.[1] On the other hand, it has been proposed that cytokines, such as TNF-α and IL-1β, stimulate DNA binding of HIF-1α, confirming the tight link between inflammation and hypoxia-inducible responses.[11]

HIF-1 is the key regulator of hypoxia-inducible genes, including the proangiogenic vascular endothelial growth factor (VEGF), as well as other genes involved in cell survival, glucose metabolism and tumour invasion.[12] The heterodimeric HIF-1 is composed of an oxygen-sensitive HIF-1α, which is strongly upregulated under hypoxic conditions, and a constitutive HIF-1β subunit. Recently, we have shown that hypoxia induces HIF-1α accumulation along with overexpression of the protease-activated receptor (PAR)-1[13], the prototypical thrombin receptor involved in inflammation and tumour progression[14], with important implications in breast cancer cell survival and migration under hypoxic conditions. Besides hypoxia, several growth factors, including thrombin, influence the levels of HIF-1α[15] in a non-hypoxic condition and, more interestingly, HIF-1α expression is positively regulated by pro-inflammatory cytokines, such IL-1β.[11] However, understanding the functional relevance of the non-hypoxic regulation of HIF-1 by IL-1 is still limited. Specifically, the role of HIF-1α in IL-1-induced migratory properties in tumour cell is still uncertain. Here, using the highly invasive human breast cancer cell line MDAMB231[16], we studied the effect of IL-1β on tumour cell migration along with HIF-1α accumulation. This model was chosen because the etiology of breast cancer is associated with inflammatory processes.[17] We show for the first time that IL-1 positively regulates the accumulation of HIF-1α with important implications for the migratory capabilities of MDAMB231 breast cancer cells.

2. Materials and Methods

2.1. Cell culture

The highly invasive breast cancer cells MDAMB231 (obtained from NCI-Frederick Cancer DCT tumour repository, Frederick, MD) were grown in RPMI (Euroclone, Devon, UK) supplemented with 10% FBS. For the experiments, cells were cultured in 35 mm Petri dishes containing 1 ml of medium. At the end of the experiments, cells were lysed for either RNA extraction or Western blot analysis. Specific mRNAs were analysed by quantitative RT-PCR (qRT-PCR). The A375 human melanoma-colony 3 and A375-colony 6 transduced with the gene encoding for IL-1α[18] were maintained in RPMI supplemented with 10% FBS. Conditioned medium (CM) was obtained from cell monolayer incubated for 48 h in serum-free medium. Supernatants were collected, centrifuged at 2000 *g* and frozen at -80°C before using.

Recombinant human IL-1α and IL-1β were obtained from Pierce Endogen (Rockford, IL) and used at different concentrations (50, 100, 300, 500 pg/ml). Tumour cell proliferation was performed by CyQUANT assay (Molecular Probe, Eugene, OR) in the absence or in the presence of IL-1β (300 pg/ml).

2.2. Migration assay

MDAMB231 cell migration was evaluated using modified Boyden chambers (Neuro Probe, Gaithersburg, MD) with gelatin-coated 8 μm pore size polycarbonate polyvinylpyrrolidone-free Nucleopore filters, as described.[19] After an overnight serum-starvation, a cellular suspension of 1×10^6 cells/ml in DMEM supplemented with 0.5% bovine serum albumin (BSA) was added to the upper chamber and a solution of medium supplemented with 0.5% BSA was added to the lower chamber. IL-1β (300 pg/ml) was added to the upper chamber and, immediately after, the cell migration assay was started. In some experiments, MDAMB231 cellular suspensions were pretreated for 30 minutes with different doses of the specific p38 MAPK-inhibitor SB203580 (Tocris Biosciences, Bristol, UK) before the addition of IL-1β. The cell migration assay was stopped after 6 h of incubation and cells on the upper surface of the filter were removed. Migrated cells on the lower surface were stained using DiffQuick (Merz-Dade, Düdingen, CH) and photographed by an OLYMPUS IX81 Research Microscope with a 10 X magnification. The images were processed with software Soft Imaging System (OlympusCompany, Munster, D). Cells migrated in 10 high-power fields were counted and data were expressed as number of migrated cells / field. Each assay was done in quadruplicate.

2.3. RNA preparation and qRT-PCR

HIF-1α, CXCL8, CXCR1, VEGF, PAR-1 mRNA expression was determined by qRT-PCR using an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Briefly, tumour cells (5×10^4well/ml) were incubated for 4-24 h, as described above. Total RNA was extracted using the TRI Reagent® (Ambion, Austin, TX). First-strand cDNA synthesis was performed using iScript™cDNA Synthesis Kit (Bio-Rad Laboratories). qRT-PCR was performed using iTaq™SYBR Green Supermix with ROX (Bio-Rad Laboratories) and the specific primers were designed using the PRIMER3 program (available at http://frodo.wi.mit.edu). Data were quantitatively analysed on an iQ™5 Optical System Software (Bio-Rad Laboratories). Relative quantification was done by using the $2^{-\Delta\Delta CT}$ method, as previously described[20]; β-actin was

used as housekeeping gene and results were expressed as fold increase in mRNA expression with respect to the control cells.

2.4. Elisa

CXCL8 concentration was assessed on cell-free supernatants by ELISA using commercial high-performance ELISA reagents (Euroclone). The assay did not show cross-reactivity with other cytokines. The minimum detectable dose for CXCL8 was < 25 pg/ml.

2.5. Western blot analysis

For Western blot analysis, cells were incubated as described above and were properly lysed. Aliquots (30 μg) of the extracted material were analysed by Western blotting using antibodies against p42/p44 MAPK, p38 MAPK or β-actin (Cell Signaling, Danvers, MA) or HIF-1α (BD Biosciences, San Jose, CA) or PAR-1 (Immunotech, Marseille Cedex, France) or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), as previously described.[13] Chemiluminescence was quantified using a ChemiDoc XRS apparatus and Quantity One software (Bio-Rad Laboratories).

2.6. HIF-1α inhibition by siRNA

siRNA sequences were selected according to Stealth RNA system (Invitrogen, Paisley, UK) for specific silencing of HIF-1α, as previously described.[13] The siRNA target sequences correspond to nucleotides 1028 to 1049 of human HIF-1α mRNA. The negative controls were designed with the same GC ratio without any known target to the human genome. Cells were transfected for 48 h with the Stealth RNAs at a concentration of 40 nM using Lipofectamine (Invitrogen) at 40% confluency of the cell monolayer. To determine the efficiency of siRNA, HIF-1α determination was conducted at both RNA and protein levels. Following transfection, experiments started accordingly with the above protocols.

2.7. Xenograft studies

For in vivo studies A375-colony 6 and A375-colony 3 (1×10^6 tumour cells) were transplanted subcutaneously in the left flank of 4-6 week old female SCID mice (Harlan, Corezzana, Italy). Procedures involving animals and their care were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies. Tumour growth was measured with a vernier caliper, and the estimates of tumour weights (mg = mm^3) were calculated as follows: (length [mm] x width [mm] 2) / 2. Mice bearing tumours of 70-80 mg were randomised (5 mice / group) to receive a cell suspension of MDAMB231 (1×10^6 cells) subcutaneously in the controlateral right flank. MDAMB231 xenografts were harvested when both A375-colony 6 and A375-colony 3 reached a tumour weight of approximately 1 g. Freshly removed tumours were finely minced, immediately frozen in liquid nitrogen and further lysed in RIPA buffer. Samples were incubated on ice for 30 minutes, sonicated and cleared by centrifugation, and then analysed by Western blot, as described above.

3. Results

3.1. IL-1β affects signalling and migratory properties in MDAMB231

A number of studies have shown that IL-1β ligation is followed by the activation of pro-inflammatory pathways associated with cell migration in several cell types.[2,21] To test this hypothesis, we here investigated whether IL-1β could activate cell migration in MDAMB231 cells. Fig. 1A shows that after a 6-h incubation, the number of migrated cells was significantly increased in the presence of IL-1β ($p < 0.05$). In contrast, IL-1β did not affect cell proliferation (data not shown), suggesting that this pro-inflammatory cytokine activates only a pro-migratory and not a proliferative programme in MDAMB231 cells. In line with the role of pro-inflammatory cytokines in tumour progression, we next determined whether IL-1β may induce the release of chemokines involved in the migration of tumour cells and in the recruitment of inflammatory cells at the tumour site. Fig. 1B shows that treatment of MDAMB231 cells with IL-1β caused a significant increase in the expression and in the release of CXCL8, a chemokine associated with motility. Interestingly, upon IL-1β treatment, MDAMB231 cells expressed higher mRNA and protein levels of PAR-1 (Fig. 1C), whose expression has been recently associated with tumour invasion and progression[14], as well as with breast cancer cell migration.[13] The overall results suggest that IL-1β may potentiate MDAMB231 cell migratory capabilities.

To identify the pathways activated by IL-1β in MDAMB231 cells, we next evaluated p38 and p42/p44 MAPK phosphorylation. Fig. 2A shows that IL-1β caused an evident and sustained phosphorylation of p38 MAPK; in contrast p42/p44 MAPK phosphorylation was not affected by IL-1β treatment. This confirms our hypothesis that IL-1β is functionally relevant in MDAMB231 by activating signal transduction pathways associated with inflammation and cell migration (e.g. p38 MAPK)[21], but not with proliferation (e.g. p42/p44 MAPK). To further establish the role of p38 MAPK in IL-1β-induced cell migration, we next determined whether the p38 MAPK inhibitor SB203580 was capable of inhibiting cell migration in MDAMB231 cells in the presence of IL-1β. Fig. 2B clearly shows that SB203580 significantly inhibited IL-1β-induced cell migration in a dose-dependent manner, confirming that IL-1β ligation is followed by the activation of pro-inflammatory pathways associated with a pro-migratory programme in MDAMB231 cells.

3.2. IL-1β induces HIF-1α accumulation and HIF-1-responsive gene expression in MDAMB231

It has been recently proposed that IL-1β upregulates HIF-1α protein under aerobic conditions in lung and colon cancer cells.[11] As expected, Fig. 3A shows the increased expression of HIF-1α protein in MDAMB231 breast cells after a 4-h incubation period in the presence of different concentrations of IL-1β. Such increase was still evident after 8-h treatment (Fig. 3B). Thus, IL-1β induces HIF-1α accumulation under aerobic conditions also in breast cancer cells. Since HIF-1α is the key-regulator of hypoxia inducible genes, associated with cell migration and angiogenesis, we next investigated whether VEGF and other HIF-1-responsive genes were upregulated in

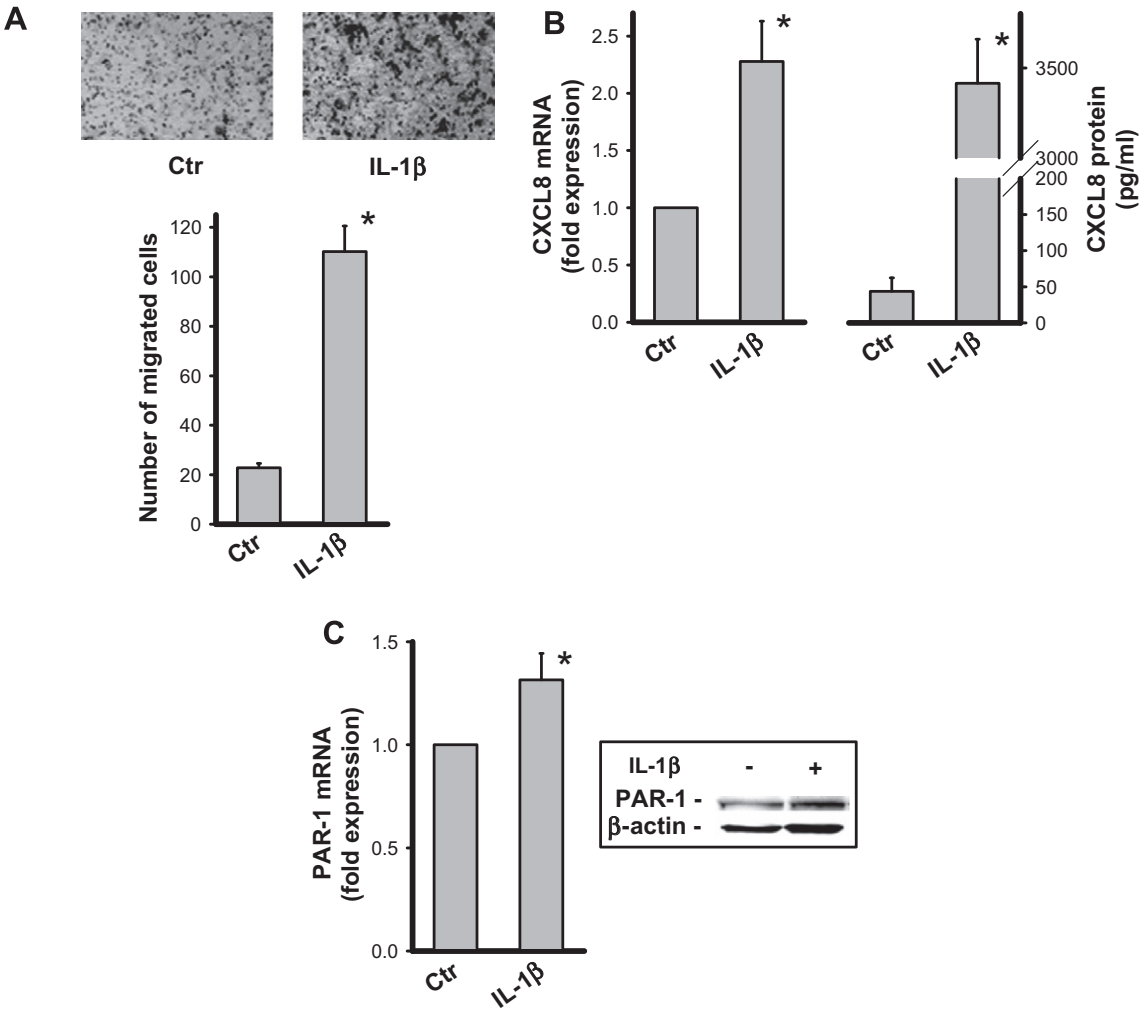

Fig. 1 – IL-1β activates a migratory programme in MDAMB231. Panel A: Cell migration was evaluated by chemotaxis microchamber technique. After a 6-h incubation, in the presence or in the absence of IL-1β (300 pg/ml), migrated cells were photographed and counted. *Top*: photomicrographs of representative fields from the indicated conditions. *Bottom*: quantification of migration using cell counts of representative fields. Results are mean ± SD of four experiments. *, significantly different from control (Ctr), $p < 0.05$ by Student's t test. Panels B-C: MDAMB231 cells were incubated for 8 or 24 h in the presence or in the absence of IL-1β (300 pg/ml) and CXCL8 (B) and PAR-1 (C) mRNA and protein expression were analysed by qRT-PCR and ELISA or Western blot. qRT-PCR and ELISA values (mean ± S.D.) refer to at least three independent experiments. *, significantly different from control (Ctr), $p < 0.05$ by Student's t test. Western blot analysis was conducted using anti-PAR-1 antibody and anti-β-actin antibody as protein loading control. A representative blot from three similar experiments is shown.

MDAMB231 cells treated with IL-1β under aerobic conditions. As shown in Fig. 3C, IL-1β treatment caused a significant upregulation of CXCR1 and VEGF mRNA expression, as determined by qRT-PCR. Of note, CXCR1 specifically mediates the biological effects of CXCL8 and is actively involved in angiogenesis and cell migration.[22] Taken together, these results suggest that IL-1β induces HIF-1-responsive gene expression under aerobic conditions in MDAMB231 cells.

3.3. *Role of HIF-1 in IL-1β-induced migratory properties of MDAMB231 cells*

As IL-1β treatment resulted in an increased migratory activity along with HIF-1α accumulation, we decided to clarify the role of HIF-1α in this phenomenon. To this end, HIF-1α expression was inhibited by siRNA and mRNA expression was analysed in MDAMB231 cells cultured in the presence of IL-1β (Fig. 4A). Treatment with Stealth siRNA resulted in a significant downregulation of HIF-1α mRNA expression (more than 80%) in IL-1β-treated cells, when compared with the Stealth control. As expected, after siRNA treatment IL-1β did not induce the accumulation of HIF-1α protein, as shown by Western blot analysis. Furthermore, when HIF-1α was inhibited by siRNA we observed a downregulation of IL-1β-induced CXCR1, as well as of VEGF, mRNA expression (Fig. 4B). More interestingly, when HIF-1α was downregulated by siRNA, a significant inhibition of IL-1β-induced migration in MDAMB231 cells was observed (Fig. 5). Thus, HIF-1α appears to be critically involved in IL-1β-induced migratory properties of MDAMB231 breast cancer cells.

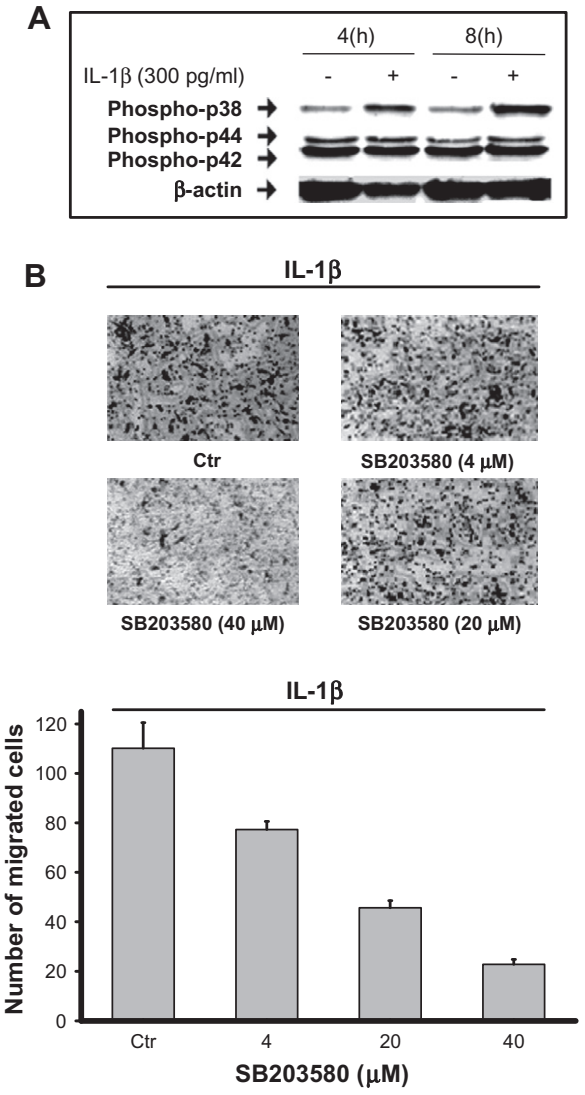

Fig. 2 – IL-1β induces p38 MAPK phosphorylation. Panel A: MDAMB231 cells were treated with IL-1β (300 pg/ml) and lysed at 4 and 8 h. Western blot analysis was conducted using either antiphospho-p38 or anti-phospho-p42/p44 MAPK antibodies. Anti-β-actin antibody was used as protein loading control. A representative blot from three similar experiments is shown. Panel B: Cell migration was evaluated by chemotaxis microchamber technique in MDAMB231 cells pretreated for 30 minutes with different doses of SB203580 before adding IL-1β (300 pg/ml). After a 6-h incubation, migrated cells were photographed and counted. *Top*: photomicrographs of representative fields from the indicated conditions. *Bottom*: quantification of migration using cell counts of representative fields. Results are mean ± SD of experiments performed in quadruplicate and repeated twice.

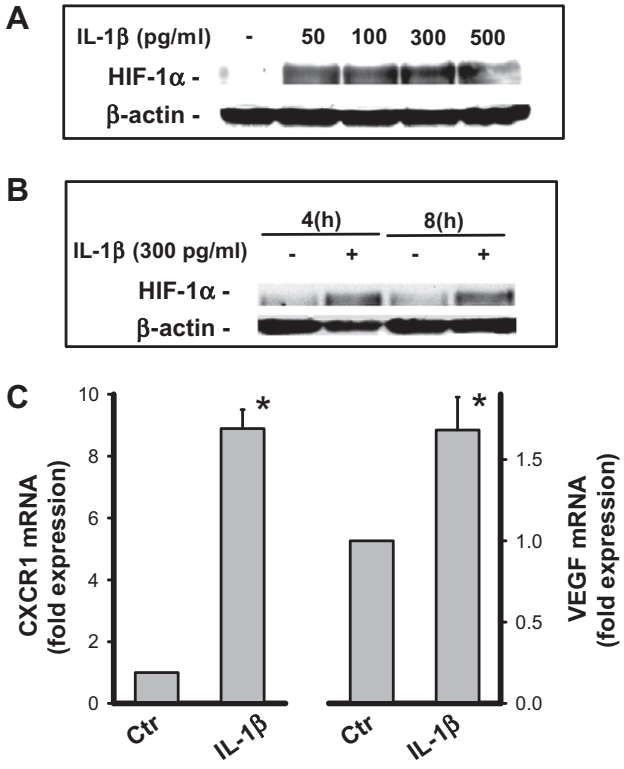

Fig. 3 – IL-1β induces HIF-1α accumulation and HIF-1-responsive gene expression. Panels A-B: MDAMB231 cells were exposed to different concentrations of IL-1β for 4 h (A) or to a single dose of IL-1β (300 pg/ml) at different time points (B). Cells were lysed and Western blot analysis was conducted using anti-HIF-1α and anti-β-actin antibodies. Representative blots from at least three similar experiments are shown. Panel C: MDAMB231 cells were incubated for 8 h in the presence or in the absence of IL-1β (300 pg/ml). CXCR1 and VEGF mRNA expression were examined by qRT-PCR. Results were obtained from three to six independent experiments and expressed as mean ± S.D. *, significantly different from control (Ctr), $p < 0.05$ by Student's t test.

3.4. *Tumour cell-derived IL-1 induces HIF-1 accumulation in MDAMB231 growing in vivo*

To test whether tumour-produced IL-1 was able to induce HIF-1 in breast cancer cells, we took advantage of the tumour models A375-colony 3 and A375-colony 6, which, respectively, does not and does express and release IL-1α.[18] In general, it is well accepted that IL-1α and IL-1β induce the same biological response[2] and only in some biological systems the two cytokines differ.[23] As expected, Fig. 6A shows that IL-1α was capable of inducing HIF-1α accumulation in MDAMB231 cells, at similar extent as IL-1β. Thereafter, we demonstrated that CM from A375-colony 6 (secreting high levels of IL-1α), but not from A375-colony 3 (not secreting IL-1α), was able to induce HIF-1 accumulation in MDAMB231 cells (Fig. 6B).

Having found that CM from A375-colony 6 induced HIF-1α in breast carcinoma cells, we studied the ability of A375-colony 6 to induce HIF-1α in MDAMB231 transplanted in nude mice. To this purpose, SCID mice bearing A375-colony 6 or A375-colony 3 subcutaneously on the left side were transplanted with MDAMB231 on the right side and left to develop for 3 weeks. Fig. 6C shows that MDAMB231 xenografts harvested from mice bearing A375-colony 6 (on the controlateral flank) have higher expression of HIF-1α when compared to tumour specimens from mice bearing A375-colony 3. No differ-

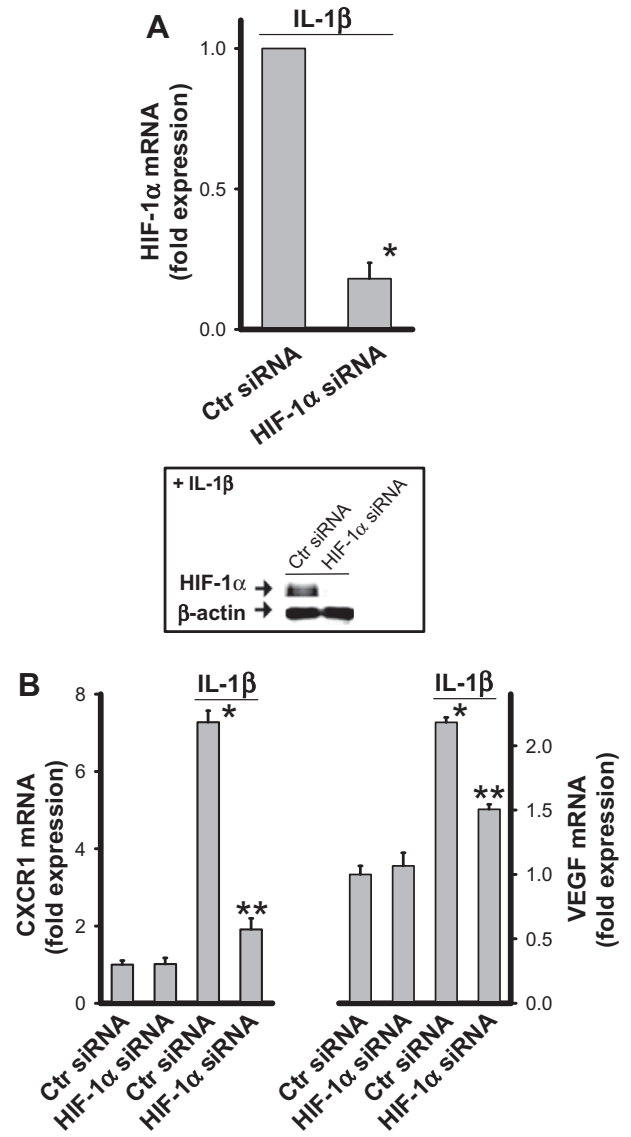

Fig. 4 – HIF-1α inhibition and HIF-1-responsive gene expression. MDAMB231 cells were treated with Stealth oligos for HIF-1α (HIF-1α siRNA) or with Stealth control oligos (Ctr siRNA) and then incubated in the presence or in the absence of IL-1β (300 pg/ml) for 8 h and analysed. Panel A: HIF-1α mRNA inhibition in IL-1β-treated MDAMB231 was quantified by qRT-PCR *(Top)*. In parallel, cellular extracts from IL-1β-treated MDAMB231 were analysed by Western blot *(Bottom)* using antibodies anti-HIF-1α and anti-β-actin as protein loading control. Representative blots from three similar experiments are shown. Panel B: CXCR1 and VEGF mRNA expression were examined by qRT-PCR and the results were expressed as described above. Values (mean ± S.D.) refer to three independent experiments. *, significantly different from Ctr siRNA in the absence of IL-1β; **, significantly different from control (Ctr siRNA) in the presence of IL-1β, $p < 0.05$ by Student's t test.

ence in tumour growth of MDAMB231 transplanted in mice bearing A375-colony 6 or A375-colony 3 was observed (data not shown). These findings indicate that IL-1 is able to induce HIF-1α accumulation also in MDAMB231 growing in vivo.

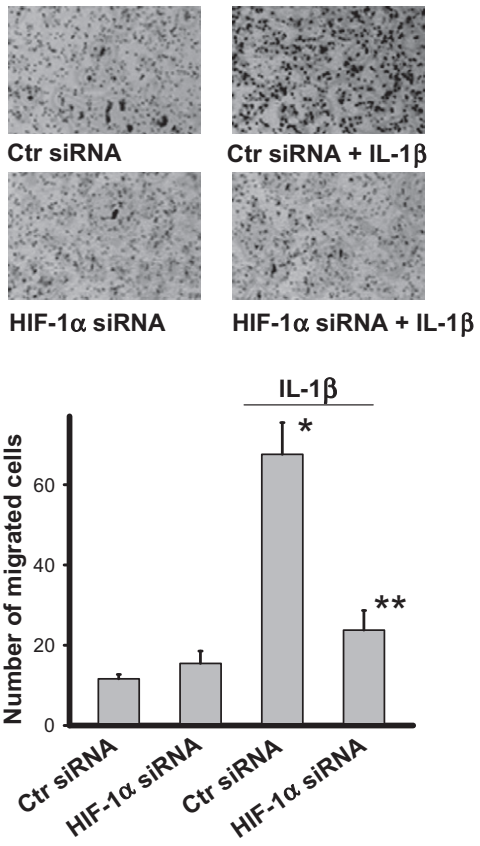

Fig. 5 – Role of HIF-1α in IL-1β-induced cell migration. MDAMB231 cells were treated with Stealth oligos for HIF-1α (HIF-1α siRNA) or with Stealth control oligos (Ctr siRNA) and then cell migration was evaluated by chemotaxis microchamber assay for 6 h in the presence or in the absence of IL-1β (300 pg/ml). *Top*: photomicrographs of representative fields from the indicated conditions. *Bottom*: quantification of migration using cell counts of representative fields. Results are mean ± SD of four experiments. *, significantly different from Ctr siRNA in the absence of IL-1β; **, significantly different from control (Ctr siRNA) in the presence of IL-1β, $p < 0.05$ by Student's t test.

4. Discussion

Previous reports have shown that IL-1 promotes HIF-1α accumulation and enhances the expression of HIF-1-responsive genes, such as VEGF, in tumour cells under aerobic conditions with important implications in tumour angiogenesis.[11] In the present study, we demonstrate for the first time that non-hypoxic induction of HIF-1α by IL-1 is involved in cancer cell migration in the model of MDAMB231 breast carcinoma.

Growing evidence indicates that inflammatory cytokines and chemokines, which can be produced by tumour cells and tumour-associated leucocytes and platelets, may contribute directly to malignant progression.[1] Particularly, host-derived IL-1β is critical for tumour growth, angiogenesis and invasiveness[5] and the delivery of IL-1 receptor antagonist reduces angiogenesis, inhibits tumour development[24] and metastasis.[25] It is widely recognised that IL-1β ligation is followed by the activation of pro-inflammatory pathways in sev-

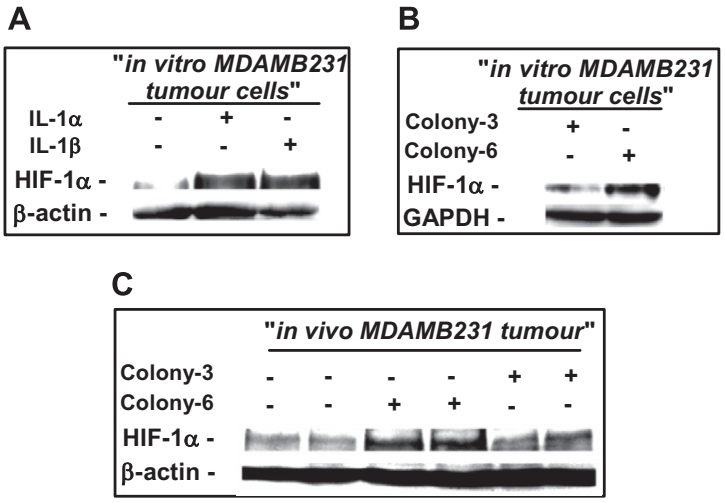

Fig. 6 – IL-1α induces HIF-1α accumulation in MDAMB231 growing in vivo. Panel A: MDAMB231 cells were exposed to IL-1α or IL-1β (300 pg/ml) for 4 h. Cells were lysed and Western blot analysis was conducted using anti-HIF-1α and anti-β-actin antibodies. A representative blot from four similar experiments is shown. Panel B: MDAMB231cells were exposed to CM from A375-colony 6 (releasing IL-1α) or A375-colony 3 (not releasing IL-1α) for 4 h. Cells were lysed and Western blot analysis was conducted using anti-HIF-1α and anti-GAPDH antibodies. A representative blot from three similar experiments is shown. Panel C: MDAMB231 tumours growing subcutaneously (2 weeks after transplantation, average size: 300 mg) were harvested from SCID mice bearing A375-colony 6 (expressing IL-1α) or A375-colony 3 (not expressing IL-1α) derived tumours onto the controlateral flank. MDAMB231 tumour specimens were lysed and Western blot analysis conducted using anti-HIF-1α and anti- β-actin antibodies. Representative blots of 2 tumours out of 5 harvested for each group; study was repeated twice.

eral cell types[2,21], including the mammalian stress-activated p38 MAPK.[26] Of interest, p38 MAPK has been recently indicated as a key signalling molecule for H-Ras-induced cell motility and invasive phenotype in human breast epithelial cells.[27] In the present study, we show that IL-1β induces p38 MAPK phosphorylation and cell migration in the highly invasive breast cancer cell line, MDAMB231, supporting the hypothesis that this pro-inflammatory cytokine may activate a pro-inflammatory pathway associated with a pro-migratory phenotype. Our study shows that IL-1β-induced cell migration was accompanied by an overexpression of PAR-1, the prototypical thrombin receptor[28], whose expression has been recently associated with tumour angiogenesis and motility/invasion.[29,30] PAR-1 is also well known for its role in several pro-inflammatory processes[28] and we and others have previously shown that PAR-1 activation resulted in the upregulation of pro-inflammatory cytokines and chemokines, such as IL-1β and CXCL8.[31] More recently we have reported that PAR-1 plays a functional role in hypoxic breast cancers cells, since its expression and activation by specific agonists resulted in an increased cell migration.[13] Thus, PAR-1 may be one of the key molecules involved in the mechanisms activated by IL-1β in tumour angiogenesis and motility/invasiveness.

Furthermore, we found that IL-1β treatment resulted in the upregulation of the specific CXCL8 receptor, CXCR1, which is transcriptionally regulated by HIF-1.[9] Of note, CXCR1/CXCL8 interaction has been shown to be critically involved in cell migration in different cell types, including breast cancer cells.[32] In addition, CXCL8 plays an integral role in promoting the malignant phenotype in breast cancer, and its production is directly influenced by inflammatory cytokines in the tumour microenvironment.[8] Indeed, IL-1β induces the expression of several pro-angiogenic cytokines and chemokines, such as CXCL8[33], and stabilisation of CXCL8 mRNA by IL-1β in malignant breast cancer cells suggests the involvement of the p38/MAPK.[8] Accordingly, we here show that IL-1β upregulates the expression and the production of CXCL8 in MDAMB231, in agreement with our present results showing an enhanced IL-1β-induced cell migration, which was reversed by p38 MAPK inhibitor SB203580. In our study, CXCR1 overexpression was associated with the upregulation of other HIF-responsive genes, such as the angiogenic cytokine VEGF[12], thus confirming the tight relationship between inflammation and HIF/hypoxia/angiogenesis in the tumour microenvironment.[1] Such relationship is also confirmed by our results, showing that IL-1β upregulates HIF-1α protein accumulation under aerobic conditions, with significant upregulation of VEGF in MDAMB231 cells as well, with important implications in breast cancer progression and angiogenesis. Our results are in agreement with previous reports, showing that IL-1β stimulates the HIF-1 signalling pathway and activates the HIF-1-responsive gene VEGF in normoxic lung carcinoma cells.[11]

The non-hypoxic upregulation of HIF-1 by IL-1β is particularly important for tumour progression and angiogenesis. It has been proposed that IL-1β-mediated upregulation of HIF-1α in cancer cells identifies HIF-1 as a critical link between inflammation and oncogenesis.[11] However, while some of the molecular mechanisms that lead to the accumulation of HIF-1 in cancer cells have been identified[11], the downstream biological effects need to be further addressed. In the present study, we identified a novel significance for the HIF-1 accumulation induced by IL-1β. Accordingly, we report that inhibition of HIF-1 resulted in the downregulation of the HIF-responsive

gene CXCR1 expression and, more interestingly, of tumour cell migration induced by IL-1β under aerobic conditions. Thus, HIF-1 appears to be one of the key molecules involved in cancer cell migration promoted by IL-1β. Several studies support the hypothesis that HIF-1 accumulation correlates with tumour progression and angiogenesis, as well as with tumour invasiveness.[12]

Finally, our observation that IL-1 induces HIF-1 accumulation in MDAMB231 cells was confirmed also in tumour cells growing in vivo using an experimental approach, mimicking the endogenous release of IL-1 in mice bearing MDAMB231 xenografts. Herein we show that HIF-1 accumulation was consistently higher in MDAMB231 tumours harvested from mice in which high level of IL-1α was secreted, indicating that the mere presence of the tumour, not expressing IL-1α, was not able to affect HIF-1 accumulation. Our results may have important implications in tumour biology, since the tumour microenvironment is characterised by the presence of cells secreting pro-inflammatory mediators, such as IL-1, capable of inducing HIF-1 accumulation.[1] Accordingly, we show that HIF-1 also accumulated in vivo in the presence of endogenously produced IL-1. This is particularly relevant in light of the recent reports, underlining the relevance of inflammation and HIF-1 in promoting tumour invasion and describing that secreted IL-1α induces a metastatic phenotype in pancreatic cancer.[34] Our *in vivo* data, along with the result that inhibition of HIF-1 led to a decrease of IL-1β-promoted cell migration further support the possible link between inflammation and cancer. The overall results may have important implications in those therapeutic approaches aimed to inhibit IL-1-mediated activities in tumour cells, specifically in breast cancer.

Conflict of interest statement

None declared.

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