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Hyaluronan induces migration of multidrug-resistant lymphoma cell lines *in vitro* through Tiam1 activation by a PI3K-dependent mechanism

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ABSTRACT

Hyaluronan (HA) modulates multidrug resistance (MDR) as well as cell migration. Tiam1 is involved in cytoskeleton reorganization during tumor invasion. In this report we show the relationship among HA, Tiam1, migration and MDR in murine lymphoma cell lines. We observed that MDR cells presented higher migratory capacity towards HA *in vitro* as well as higher constitutive active Tiam1 expression than the sensitive cell line. Besides, HA treatment induced migration towards HA of MDR cell lines through Tiam1 activation by a PI3K-dependent mechanism, showing that disruption of HA signaling would be useful in treatment of MDR hematological malignancies.

1. Introduction

Multidrug resistance (MDR) constitutes a major obstacle in the treatment of refractory disease in hematological malignancies. MDR has usually been explained as a consequence of the overexpression of ATP-dependent efflux pumps such as P-glycoprotein (Pgp), reduced drug uptake, activation of detoxifying systems, or alterations in survival (overexpression of phosphatidylinositol 3-kinase-PI3K-/Akt pathway) or apoptotic pathways [1–3]. In addition, Pgp association with the actin cytoskeleton has been shown to be crucial in conferring a multidrug-resistant phenotype to human lymphoid cells, thus indicating that the cytoskeleton function is involved in drug resistance and tumor progression [4]. Besides, increasing evidence supports the role of the tumor microenvironment in conferring drug resistance as a major cause of relapse in cancers [5].

Hyaluronan (HA) is a linear glycosaminoglycan (GAG), composed of repeated disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, with a molecular weight ranging from 10^5 to 10^7 Da. HA is a conspicuous component of the mammalian extracellular matrix, where it possesses several functions both in physiological and pathological conditions such as morphogenesis, tissue injury and repair, inflammation, and tumorigenesis [6–9]. Increased levels of HA have been found in the tumor microenvironment and have been shown to promote tumor progression and metastasis [10,11]. Upon interaction with cell surface receptors like CD44, RHAMM, LYVE-1 or different HA binding-proteins (TSG-6, SHAP), HA is able to modulate fundamental cell behavior such as cell proliferation, apoptosis, adhesion, migration and multidrug resistance. CD44 is a cell surface adhesion molecule expressed in different isoforms, which have been found in many types of tumors including lymphomas and have been associated with cancer dissemination [12,13]. Interaction of CD44 with HA appears to promote cell motility since this interaction activates the ankyrin-based cytoskeleton and the Rho GTPases signaling (Rac1, Cdc42 and RhoA) leading to cellular activities such as gene transcription, cytoskeleton reorganization, cell growth and tumor cell migration [14].

Tiam1 (T-lymphoma invasion and metastasis) is a guanine nucleotide exchange factor (GEF) for Rho GTPases *in vitro* and is a specific activator of Rac1 *in vivo*, involved in membrane cytoskeleton rearrangements, cell adhesion, migration, and consequently in tumor invasion and progression [15]. Tiam1 was first identified in a screen for genes that, when amplified, are able to induce invasiveness of murine lymphoma cells [16]. However, Tiam1 signaling may affect adhesion or migration in a cell type-specific manner [17]. In fact, Tiam1 has been shown to have an important role in promoting tumor migration and invasion in breast, colon and hepatocellular

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carcinomas [18–21] as well as in lymphoma metastasis [22]. Nevertheless, in renal carcinoma cells, Tiam1 promotes adhesion and inhibits invasion [23]. Structurally, Tiam1 protein contains several functional domains including a characteristic Dbi homology (DH) domain (with the catalytic activity), a Ras-binding domain (RBD) and two pleckstrin homology (PH) domains, one at the N-terminus (N-PH) and the other at the C-terminus (C-PH). Regulation of Tiam1 occurs by phosphorylation and translocation to the membrane [15]. PI3K lipid products, via a mechanism that requires interaction with the N-PH domain, appear to be important in Tiam1 translocation to the plasma membrane and subsequent activation [24,25]. Besides, PI3K-independent mechanisms could also be involved in Tiam1 activation [26]. Studies performed in breast tumor cells have shown that Tiam1 interaction with CD44 is required for HA-stimulated Rac1 signaling and cytoskeleton-mediated tumor cell migration [14]. However, the mechanisms involving activation of Tiam1 in lymphoma cells are still unknown.

Depending on its molecular size, HA is able to modulate MDR in a variety of cancer cell types [27–31] including lymphoma cells [32]. Specifically, our group has demonstrated that HA fragments sensitize lymphoma resistant cell lines to vincristine by modulating Pgp activity and PI3K/Akt pathway [32]. Recent investigations have also shown that HA/CD44 interaction induces ankyrin (a cytoskeleton protein) binding to Pgp resulting in the efflux of chemotherapeutic drugs and chemoresistance [33]. In addition, Pgp interaction with CD44 promotes cell migration and invasion of breast tumor and melanoma cells, indicating a relationship between both processes and MDR [34,35]. Although several studies have analyzed typical mechanisms of HA-modulated MDR, little is known about the role of HA in migration of MDR lymphoma cell lines.

The aim of this work was to determine the role of HA and Tiam1 in cell migration in murine lymphoma cell lines resistant to doxorubicin (LBR-D160), vincristine (LBR-V160) and a sensitive line (LBR−). Our results showed that MDR lymphoma cell lines presented a higher migratory capacity towards HA *in vitro* as well as a higher active Tiam1 expression than the sensitive cell line. Besides, HA treatment induced migration of MDR cell lines towards HA through Tiam1 activation by a PI3K-dependent mechanism.

2. Materials and methods

2.1. Reagents

Recombinant high molecular weight (HMW, 1.5–1.8 × 10^6 Da) HA (CPN spol.s.r.o Czech Republic) was supplied by Farmatrade (Argentina). Anti-CD44 (KM81) was kindly provided by Dr. K. Mikecz (Rush University Medical Center, Chicago, USA). Wortmannin and LY294002 were purchased from Calbiochem (La Jolla, CA). Vincristine (VCR) was provided by Filaxis Pharmaceuticals S.A. Argentina and doxorubicin (DOX) by Gador Pharmaceuticals Argentina. Antibodies against Tiam1 (C-16), actin (C-10), rat IgG2a (isotype control), anti-rabbit secondary horseradish peroxidase and anti-goat secondary horseradish peroxidase were purchased from Santa Cruz Biotechnology (USA). RPMI 1640 was purchased from Invitrogen (Argentina). The plasmids GFP-C1199-Tiam1 and GFP-ΔN-PH-Tiam1 were kindly given by Dr. I. Fleming (Department of Biochemistry, University of Dundee, Scotland, UK).

2.2. Cell culture

The doxorubicin-resistant (LBR-D160), vincristine-resistant (LBR-V160) and sensitive (LBR−) murine lymphoma cell lines were obtained previously in our laboratory [36]. Cell lines were grown in suspension cultures at 37 °C in a 5% CO_2 atmosphere with RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 10 mM HEPES buffer, 5 × 10^{-5} M 2-mercaptoethanol, 100 μg/ml streptomycin and 100 μg/ml penicillin. The resistant cell lines were cultured in the presence of 160 ng/ml VCR or DOX respectively.

2.3. Migration assay

A 48 well microchemotaxis Boyden chamber (Neuroprobe, Inc., MD) with polycarbonate membranes (Nucleopore membrane, 5 μm pore size, Neuroprobe) was used. Cells (3 × 10^6 cells/ml) were placed in the upper chamber of the transwell unit while the lower wells contained 200 μg/ml HA or RPMI 1640. After incubation for 3 h at 37 °C, the membrane was removed and cells on the upper side of the membrane were scraped off with a blade. Cells attached to the lower side of the membrane were fixed in 2% formaldehyde and stained in 10% DAPI. Cells were counted in a fluorescence microscope (OLYMPUS BX51, Olympus Corporation) using a 40× objective. Ten fields per well were recorded and mean number of cells per field as well as standard error of the mean (SEM) were calculated. To evaluate the capacity of anti-mouse CD44 monoclonal antibodies to inhibit HA-mediated migration, cells were incubated with KM81 mAb (20 μg/ml) or with normal rat IgG2a (isotype control) for 30 min at 37 °C before migration assay. The ability of HA to modulate migration of these cell lines towards HA was investigated by pre-treating the cells with 200 μg/ml HA for 60 min at 37 °C before migration. Besides, the ability of PI3K inhibitors to modulate HA-mediated migration was tested incubating cells with wortmannin (0.5 μM) or LY294002 (10 μM) for 30 min at 37 °C before migration assay. Results were expressed as migration index (MI) = mean total cells per field of samples with chemoattractant/mean total cells per field of samples without chemoattractant. RPMI 1640 controls were performed in the absence of chemoattractant in the lower wells thus having average migration index close to one.

2.4. Membrane extracts and western blot

Cells were untreated or treated with 200 μg/ml HA, 20 μg/ml anti-CD44 (KM81) or anti-CD44 plus HA for 2 h. Besides, treatments with 0.5 μM wortmannin, 10 μM LY294002 or vehicle (DMSO) for 30 min were done. After treatment, cells were resuspended in lysis buffer (HEPES 10 mM pH 7.4, NaCl 38 mM, PMSF 25 μg/ml, leupeptin 1 μg/ml and aprotinin 1 μg/ml), incubated at −40 °C over night and then centrifuged at 2700 rpm for 10 min. The supernatant was then centrifuged at 33,000 rpm for 90 min. The supernatant constitutes the cytosolic/soluble extract and the pellet, the membrane/insoluble extract, which was resuspended in lysis buffer with 0.05% Triton X-100 and both aliquots were stored at −70 °C until used. Equal amounts of protein were resolved by SDS-PAGE, transferred onto a nitrocellulose membrane and then incubated with specific antibodies to Tiam1 or actin. The reaction was revealed with horseradish peroxidase-labeled secondary antibody and developed using a chemiluminescent detection system. Gel images obtained with a digital camera were subjected to densitometric analysis using Image Scion Software (Scion Corporation, USA).

2.5. Transfection experiments and confocal microscopy

Cells were transfected with GFP-tagged proteins for 24 h, and then were stimulated with HA for 10, 30 and 120 min, with or without pre-treatment with 0.5 μM wortmannin for 30 min. The plasmid and transfection conditions for expression of GFP-C1199-Tiam1 (an active form of Tiam1) and GFP-ΔN-PH-Tiam1 (a truncated form of Tiam1 with an N-PH domain deletion) have been previously described [24]. Cells were stained with propidium iodide to visualize the cellular nucleus, fixed in cold acetone for 20 min and then washed four times in PBS before mounting on slides in UltraCruz™ Mounting Medium (Santa Cruz Biotechnology, USA). The labeled cells were analyzed by laser scanning confocal microscopy using a Zeiss confocal microscope.

2.6. Statistical analysis

Statistical significance between groups was evaluated by one way-ANOVA and means were compared by the Tukey's test (migration assay) or Dunnet's test (densitometric analysis). Differences between groups were considered significant at the level of $P < 0.05$. Analysis was performed using Prism 4 software (Graph Pad, San Diego, CA, USA).

3. Results

3.1. Resistant cell lines present a higher migratory capacity towards HA in vitro as well as a higher constitutive expression of Tiam1 in membrane/insoluble extracts

To evaluate the ability of hyaluronan (HA) to act as a chemoattractant for murine lymphoma sensitive and resistant cell lines, a migration assay towards HA was performed. LBR-D160 and LBR-V160 presented a higher migration towards HA (200 μg/ml) as compared to RPMI 1640 (migration indexes of 1.46 and 1.36 *vs.* 1, respectively). However, LBR− did not show a significantly enhanced migration towards HA (migration index 1.13 *vs.* 1). Consequently, migration towards HA was found increased in LBR-D160 and LBR-V160, as compared to LBR− ($P < 0.001$ and $P < 0.01$, respectively). Besides, a similar migration capacity was observed between both resistant cell lines (Fig. 1A). It is noteworthy that LBR− presented higher migration both towards RPMI 1640 and HA than LBR-D160

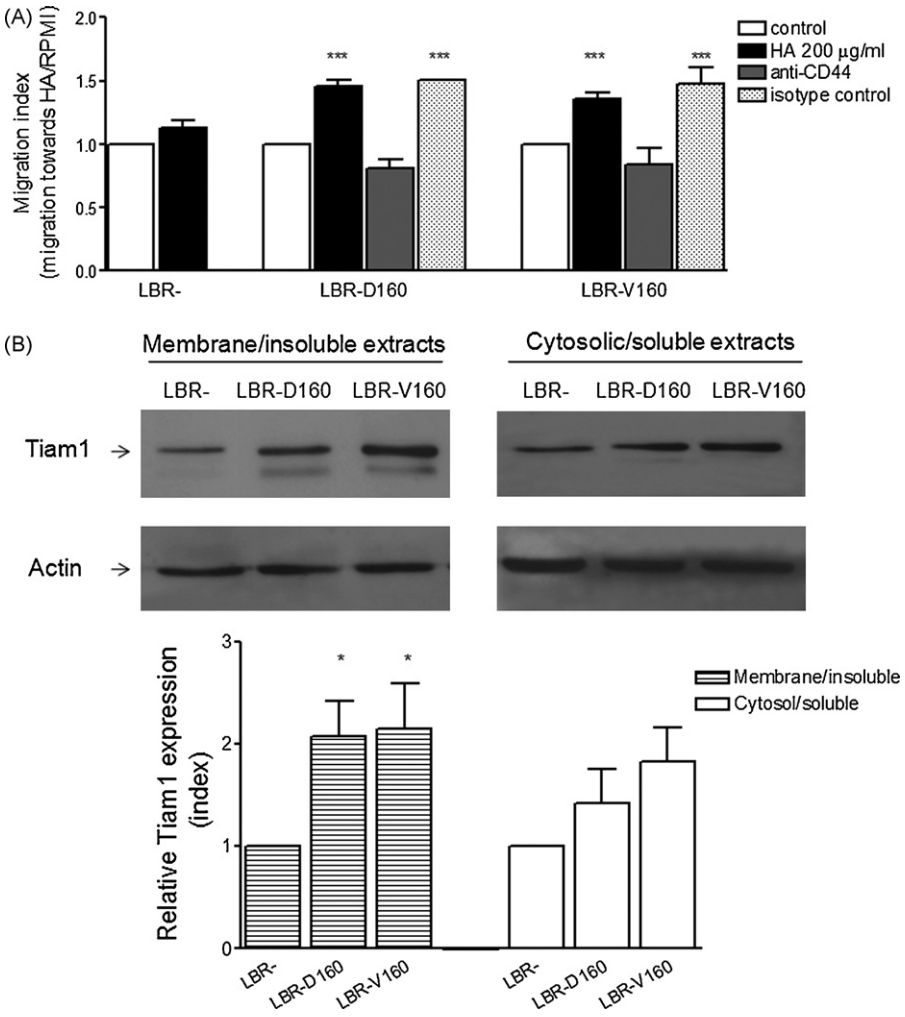

Fig. 1. Migration towards HA *in vitro* and constitutive expression of Tiam1 in LBR−, LBR-D160 and LBR-V160. (A) Migration assay of cells towards RPMI 1640 (control) or 200 μg/ml HA. Pre-treatment with either anti-CD44 KM81 antibody or rat IgG isotype control was performed. Results are expressed as migration index (migration towards HA/migration towards RPMI 1640) and are representative of three independent experiments, in quadruplicates. RPMI 1640 controls were performed in the absence of HA in the lower wells thus having average migration index close to one. Bars represent mean ± SEM. ***$P < 0.001$ *vs.* control. (B) Constitutive expression of Tiam1 in membrane/insoluble and cytosolic/soluble extracts in the three cell lines by western blot. Expression of actin was used as loading control. Similar results were obtained in three independent experiments. Densitometric analysis of the bands was performed and the results expressed as the resistant cell line/sensitive cell line index, as determined from three independent experiments. Bars represent mean ± SEM. *$P < 0.05$ *vs.* LBR−.

and LBR-V160, although migration towards HA in comparison with RPMI 1640 was not significantly increased in LBR− (Table 1). In LBR-D160 and LBR-V160 cell lines, migration towards HA was inhibited after incubating with anti-CD44 (KM 81) as compared to controls, while rat IgG isotype control did not modify migration of the cell lines (Fig. 1A). These results indicate that migration towards HA was mediated by CD44 in LBR-D160 and LBR-V160.

Since Tiam1 increases migration of several tumor cells, we next examined constitutive expression of Tiam1 in the three cell lines. Western blot analysis was performed both in membrane/insoluble and cytosolic/soluble extracts in order to evaluate active (membrane bound) and inactive (cytosolic) Tiam1. As shown in Fig. 1B, Tiam1 expression in membrane/insoluble fraction was two-folds increased in LBR-D160 and LBR-V160, as compared to LBR− ($P < 0.05$). We also analyzed Tiam1 expression in cytosolic/soluble fraction in the three cell lines, but found no significant differences.

These results show that both resistant cell lines presented a higher migration towards HA and a higher constitutive membrane Tiam1 expression (active Tiam1) than the sensitive line LBR−.

3.2. HA modulates cell migration and Tiam1 activation

Our next aim was to investigate whether HA treatment was able to modulate migration of the tumor cell lines and Tiam1 expression. First, we performed migration assays towards RPMI

Table 1
Migration of LBR−, LBR-D160 and LBR-V160 towards RPMI 1640 or 200 μg/ml HA.

	LBR−		LBR-D160		LBR-V160	
	RPMI	HA	RPMI	HA	RPMI	HA
Exp. 1	128.7 ± 5.3	149.4 ± 9.8	47.24 ± 2.6	71.9 ± 3.4	53.32 ± 3.0	76.4 ± 2.4
Exp. 2	99.2 ± 4.5	109.5 ± 4.9	44.5 ± 2.4	59.0 ± 2.4	46.4 ± 1.9	61.5 ± 3.2
Exp. 3	92.3 ± 3.2	113.1 ± 4.2	37.0 ± 2.0	58.1 ± 2.0	42.7 ± 5.4	63.8 ± 2.9

All listed data were determined as described in Section 2 and expressed as mean number of migrated cells ± SEM.

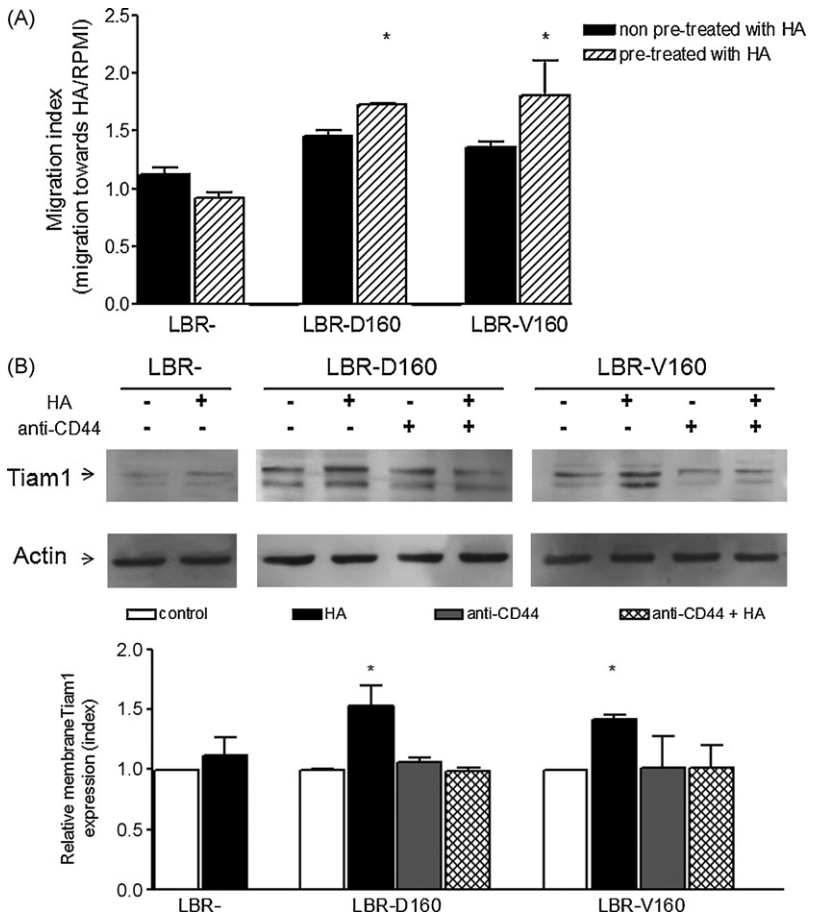

Fig. 2. Effects of HA in cell migration and Tiam1 expression in LBR−, LBR-D160 and LBR-V160. (A) Migration assay towards 200 μg/ml HA of non pre-treated cells or cells pre-treated with 200 μg/ml HA. Results are expressed as migration index (migration towards HA/migration towards RPMI 1640) and are representative of three independent experiments, in quadruplicates. Bars represent mean ± SEM. $^*P < 0.05$ *vs.* non pre-treated cell lines. (B) Tiam1 expression in the three cell lines. Membrane/insoluble extracts from untreated cells and cells treated with 200 μg/ml HA, 20 μg/ml anti-CD44 alone or anti-CD44 plus HA for 2 h. Expression of actin was used as loading control. Similar results were obtained in three independent experiments. Densitometric analysis of the bands was performed and the results expressed as the treated cells/untreated cells index. Bars represent mean ± SEM. $^*P < 0.05$ *vs.* untreated.

1640 and HA of the different cell lines with or without HA pre-treatment. After HA pre-treatment, we observed that LBR-D160 and LBR-V160 presented a significantly increased migratory capacity towards HA as compared to LBR− (migration indexes of 1.73 and 1.70 *vs.* 0.90, $P < 0.001$, respectively). Although migration towards HA of LBR-D160 and LBR-V160 pre-treated cells compared with non pre-treated cells was slightly increased (18% and 32% increase, respectively), this was statistically significant (Fig. 2A).

Then, we investigated whether HA was able to modulate Tiam1 expression. For this purpose, membrane/insoluble and cytosolic/soluble extracts from the different cell lines either treated or not with 200 μg/ml HA were prepared and western blot analysis was performed. HA treatment induced an increase in Tiam1 expression in the membrane/insoluble fraction in LBR-D160 and LBR-V160, but not in LBR− (Fig. 2B). Indeed, after HA treatment membrane Tiam1 was observed 50% and 40% increased in LBR-D160 and LBR-V160, respectively. Besides, Tiam1 expression was also evaluated in the cytosolic/soluble fraction and no significant changes were found in Tiam1 levels after HA treatment in any of the cell lines studied (data not shown). To evaluate whether the effect of HA on Tiam1 expression was mediated by the interaction with CD44, resistant cell lines were treated with anti-CD44 plus HA. Co-treatment with anti-CD44 abolished the effect of HA, being the expression of Tiam1 in the membrane/insoluble fraction similar to that observed in the untreated cells (Fig. 2B). Besides, no changes were observed in membrane Tiam1 expression with anti-CD44 alone.

In order to confirm the ability of HA to induce Tiam1 activation in the resistant cell lines, LBR-V160 was transfected with GFP-C1199-Tiam1 (a plasmid able to express a functional Tiam1 protein) and treated with HA for 10, 30 and 120 min, and cellular distribution of GFP-C1199-Tiam1 was analyzed by confocal microscopy. In non-stimulated cells, GFP-C1199-Tiam1 was distributed both in the cytosol and plasma membrane (Fig. 3A). However, the addition of 200 μg/ml HA induced a rapid (within 10 min) translocation of C1199-Tiam1 from the cytosol to the plasma membrane which was maintained even 120 min later, demonstrating that HA induces Tiam1 activation in LBR-V160.

To sum up, our results indicate that HA is able to induce Tiam1 activation in the resistant cell lines, thus leading to their migration towards HA *in vitro,* an effect mediated by the CD44 receptor.

3.3. Inhibition of the PI3K signaling pathway decreases migration towards HA and Tiam1 activation in resistant cell lines

Since we have previously demonstrated that HA can activate the PI3K pathway [32,37] and that HA is able to increase Tiam1 activation, we next evaluated whether HA effects in Tiam1 activation were mediated by the PI3K pathway.

First, we investigated the effect of PI3K inhibition on migration towards HA *in vitro* and found that PI3K inhibitor wortmannin decreased migration of LBR-D160 and LBR-V160 towards HA (48% and 27%, respectively). Besides, treatment with LY294002 (another

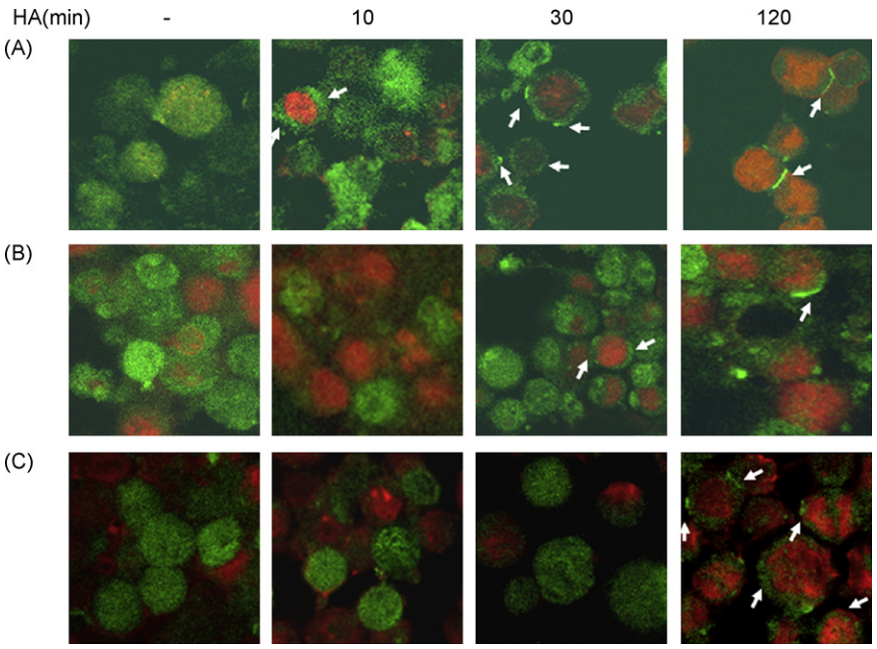

Fig. 3. Analysis of exogen Tiam1 subcelullar distribution by confocal microscopy. LBR-V160 cell line was transfected with GFP-C1199-Tiam1 (A and B) or GFP-ΔN-PH-Tiam1 (C) and then stimulated with 200 μg/ml HA for 10, 30 and 120 min. (B) Cells were pre-treated with 0.5 μM wortmannin for 30 min. Red staining allows visualization of the cellular nucleus. The arrows indicate Tiam1 translocation to the cell membrane. Results are representative of three independent experiments.

PI3K inhibitor) also induced a significant decrease in migration of both resistant cell lines towards HA *in vitro* (35% and 50%, respectively) (Fig. 4A).

Then, we determined Tiam1 expression in membrane/insoluble and cytosolic/soluble extracts after treatment with wortmannin or LY294002 by western blot. In membrane/insoluble fraction, we observed that wortmannin (0.5 μM) induced a decrease in Tiam1 expression in LBR-D160 and LBR-V160 (40% and 31%, respectively), but no changes in LBR− (3%) (Fig. 4B). We also found that LY294002 significantly decreased membrane Tiam1 expression in LBR-D160 and LBR-V160 (39% and 38%, respectively) but not in LBR− (Fig. 4B). Besides, neither wortmannin nor LY294002 were able to modulate cytosolic Tiam1 expression in any of the cell lines tested (data not shown).

Finally, we evaluated the effect of PI3K/Akt inhibition in HA-induced Tiam1 activation by confocal microscopy. For this purpose, two strategies were carried out. First, the LBR-V160 cell line was transfected with GFP-C1199-Tiam1 for 24 h, pre-treated with wortmannin (0.5 μM) for 30 min and then stimulated with HA for 10, 30 and 120 min. We found that translocation of Tiam1 to the plasma membrane was reached only at 30 and 120 min after HA treatment (Fig. 3B), showing a delay in comparison with cells not pre-treated with wortmannin (Fig. 3A). Second, LBR-V160 was transfected with GFP-ΔN-PH-Tiam1 (a construct with an N-PH domain deletion which is an important site for binding to phosphoinositides and plasma membrane anchorage) and then treated with HA. Although the PI3K-dependent mechanism of Tiam1 translocation was inhibited, HA was also able to induce translocation of GFP-ΔN-PH-Tiam1 to the plasma membrane but only 120 min after the treatment (Fig. 3C). These results suggest that HA is able to induce activation of Tiam1 (translocation to the plasma membrane) in the resistant cell lines partly mediated by a PI3K-dependent mechanism and partly by a PI3K-independent mechanism, since translocation of Tiam1 was delayed but not abolished by PI3K inhibition or mutation of N-PH-Tiam1 domain.

Taken together, our results suggest that inhibition of PI3K seems to decrease the migratory capacity towards HA in the resistant cell lines by delaying Tiam1 activation.

4. Discussion

The main causes of treatment failure in cancer are MDR and development of metastasis. Classically, these processes have been separately studied. Although in the last few years their connection has started to be evaluated, the involvement of the tumor microenvironment component HA and its relationship with MDR and metastasis is still unclear.

In this work, we demonstrated that both resistant cell lines studied (LBR-D160 and LBR-V160) presented a higher migration towards HA and a higher constitutive membrane Tiam1 expression (active Tiam1) than the sensitive line LBR−. Despite being migration towards HA and media alone (RPMI 1640) similar in LBR−, this cell line presented higher number of migratory cells towards RPMI 1640 than LBR-D160 and LBR-V160. A direct correlation between the metastatic potential and the MDR phenotype has been previously observed in melanoma cells [38]. Besides, it has been demonstrated that MDR hepatoma cells (Pgp+) display an increased capacity to migrate in comparison with Pgp− parental cells [39]. Moreover, Pgp substrates seem to be able to induce membrane ruffling, an early indicator of cell motility and metastatic potential, in Pgp+ cells by a PI3K-dependent mechanism [40]. We showed that MDR lymphoma cell lines (LBR-D160 and LBR-V160) presented a decreased constitutive migratory capacity (towards RPMI 1640) than the sensitive lymphoma cell line (LBR−). However, both lymphoma resistant cell lines LBR-D160 and LBR-V160 – Pgp+ cell lines [36] – presented a higher migratory capacity towards HA *in vitro* than the sensitive Pgp− cell line indicating that expression of Pgp would be involved in migration towards HA. We have previously reported that LBR− (Pgp− cell line which is grown without drug pressure) possesses the major invasive capacity *in vivo* [42]. Although LBR− migration capacity *in vitro* and its metastatic behavior *in vivo* are higher than those of LBR-D160 and LBR-V160, our results indicate that LBR− does not present a preferential migration capacity towards HA. Therefore, the relevance of our present work resides in the fact that MDR lymphoma cells are able to respond to HA (a component of the tumor microenvironment that can induce several changes in cell behavior) by presenting an enhanced

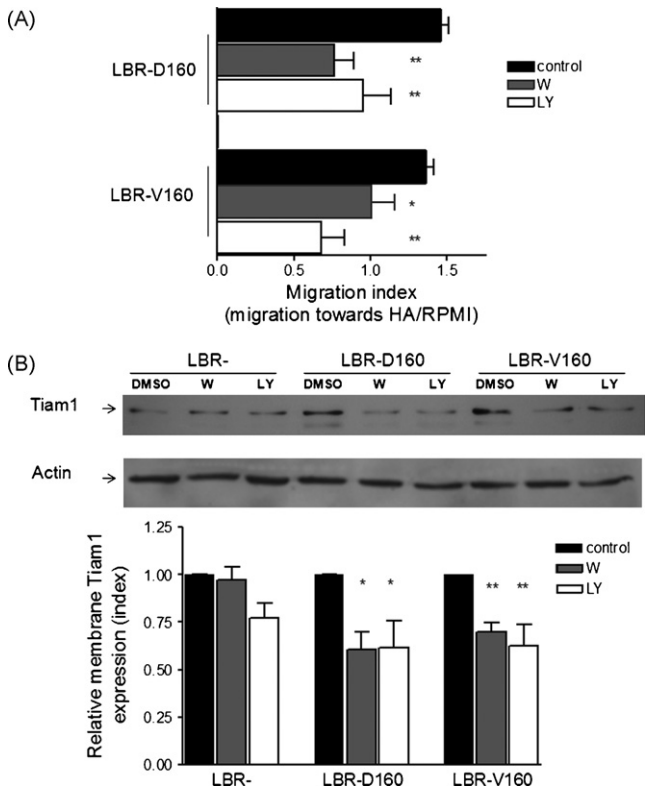

Fig. 4. Effects of PI3K inhibition in migration and Tiam1 expression in LBR−, LBR-D160 and LBR-V160. (A) Migration assay of cells pre-treated with wortmannin (0.5 μM), LY294002 (10 μM) or DMSO (control) towards RPMI 1640 or 200 μg/ml HA. Results are expressed as migration index (migration towards HA/migration towards RPMI 1640) and are representative of three independent experiments, in quadruplicates. Bars represent mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* control. (B) Tiam1 expression from the three cell lines after treatment with PI3K inhibitors. Membrane/insoluble extracts from untreated cells (DMSO) and cells treated with 0.5 μM wortmannin (W) or 10 μM LY294002 (LY). Expression of actin was used as loading control. Similar results were obtained in three independent experiments. Densitometric analysis of the bands was performed and the results expressed as the treated cells/untreated cells index. Bars represent mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs.* untreated.

migration towards it, whereas the sensitive cell line failed. Further experiments evaluating migration towards other ECM components are required to clarify the relationship between MDR and migration.

Furthermore, migration towards HA in LBR-D160 and LBR-V160 was mediated by CD44, the main cell surface receptor for HA. CD44 involvement in invasion and metastasis has been studied even in lymphomas [13]. Besides, some recent reports have linked invasion with MDR based on the promotion of cell migration and invasion by CD44/Pgp interaction in colon carcinoma, breast tumor and melanoma cells [34,35,41]. Moreover, Miletti-Gonzalez et al. have found that inhibition of CD44 or Pgp function reduces both migration and invasion of those cells [34]. We have previously demonstrated that the three cell lines present a similar expression of CD44 [42]. In this work, we report a novel connection between HA/CD44 interaction and MDR in lymphoma cell lines based on CD44 ability to preferentially induce migration towards HA of Pgp+ cells (LBR-D160 and LBR-V160).

In this work we clearly demonstrated CD44-dependent HA effects, however our group has previously reported that these cell lines failed to bind HA [42]. It is well known that binding of soluble HA to the cell surface is a complex interaction of multivalent binding events affected by the size of HA, the quantity and distribution of cell surface HA receptors (mainly CD44), and the activation state of CD44 [43]. Thus, the absence of constitutive cell binding to soluble HA would not necessarily indicate absence of molecular HA/CD44 interaction. Indeed, Gál et al. have demonstrated that a lymphoma cell line – which showed no binding to soluble HA and very poor binding to immobilized HA – was able to roll on an HA substrate with a higher rolling velocity than those cells that presented high binding to HA, suggesting a greater migration capacity [44]. Besides, it has been demonstrated that endogenous HA synthesis is able to interfere with HA binding to the cell surface [45]. Since we have recently found that these cell lines synthesize HA (unpublished data), this finding may also explain why we did not detect HA binding when there was at least a weak binding to HA—which would be enough to induce the effects observed in the present work. Moreover, we have also reported that HA fragments are able to bind to these cells by CD44 inducing cellular signaling pathways [32] probably by displacing endogenous HA or by direct interaction with its receptor [9,32,46].

Our findings also show that both resistant cell lines presented a higher constitutive active Tiam1 expression than the sensitive one. Previous studies have demonstrated that up-regulation of Tiam1 is correlated with an increased migration of colon tumor cells [20]. In human breast cancer, Tiam1 levels are correlated with an increased tumor grade and/or poor prognosis [18,19]. In hepatocellular carcinoma (HCC), Tiam1 expression has been found increased in a group of patients with a significantly shorter overall survival time, thus showing that Tiam1 may be a novel predictor for the prognosis of HCC patients [21]. Thus, our results lead us to hypothesize that the enhanced constitutive expression of active Tiam1 in the resistant cell lines studied could be related to the increased migration of these MDR lymphoma cells towards HA *in vitro*.

On the other hand, we investigated whether HA treatment was able to modulate migration and Tiam1 expression in MDR lymphoma cell lines. In breast tumor cells, it has been previously demonstrated that the binding of HA to CD44 promotes Tiam1-mediated Rac1 activation and cytoskeleton-mediated tumor cell migration [47]. In this work, we showed that HA is able to induce Tiam1 activation to the plasma membrane in LBR-D160 and LBR-V160 but not in LBR−. We also found that this effect was abolished with CD44 receptor inhibition. These data strongly suggest that HA/CD44 interaction is crucial for HA induction of Tiam1 activation in these MDR lymphoma cells. We also demonstrated that HA pre-treatment enhanced migration of MDR lymphoma cells towards HA *in vitro*, but not of the sensitive lymphoma cells. These findings support a role for HA in Tiam1-mediated migration of MDR lymphoma cells.

Since membrane translocation of Tiam1 – which is crucial for its activation – requires binding of PI3K lipid products to the N-PH domain [24,25], our next step was to evaluate whether HA effects in Tiam1 activation were mediated by the PI3K pathway. We found that the inhibition of PI3K signaling was able to significantly reduce migration of the resistant cell lines towards HA. Besides, PI3K inhibition also reduced Tiam1 activation in the resistant cell lines as shown by the decrease of Tiam1 in membrane/insoluble extracts observed in the western blot as well as by the delay in Tiam1 translocation in confocal microscopy analysis.

The PI3K signal transduction pathway plays a central role in oncogenesis by regulating intracellular processes such as cell survival, apoptosis, cell growth, angiogenesis, motility and chemoresistance [2]. PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3), which recruits downstream effectors (such as the kinase Akt) to the plasma membrane where they are activated. We have previously observed that the resistant cell lines present higher PI3K/Akt activity than the sensitive one [48]. HA is able to modulate the PI3K/Akt pathway in different cancer types including lymphoma [32,37]. Besides, a recent study has demonstrated a clear link between HA, PI3K and migration since stimulation of human pancreatic cancer cells by HA activated PI3K/Akt signal-

ing, thus modulating cancer cell motility and migration *in vitro* and metastasis *in vivo* [49]. In the present work we found that the lymphoma resistant cell lines – with increased PIP3 production – presented not only an increased active Tiam1 expression but also an enhanced migration towards HA *in vitro*. In addition, disruption of the PI3K signaling decreased Tiam1 localization at the plasma membrane and consequently inhibited its migration towards HA. Our results clearly show the relevance of the PI3K signaling pathway in Tiam1 activation and migration towards HA in MDR lymphoma cells. However, it has been recently reported that Tiam1 deficiency increases aggressiveness and infiltration of PI3K-induced lymphomas [50]. In contrast, our results suggest that the presence of Tiam1 increases migration towards HA and subsequent invasion in MDR lymphoma cells with up-regulated PI3K/Akt pathway. These discrepancies could be due to the differences in the tumor models used in each study.

It is noteworthy that inhibition of the PI3K pathway delayed but did not abolish Tiam1 translocation to the plasma membrane, as shown by confocal microscopy analysis. These data suggest that the activation of Tiam1 is mediated by PI3K-dependent as well as by PI3K-independent mechanisms. As previously reported, Ras can induce Tiam1 activation through its direct interaction with the RBD domain in Tiam1 protein and can promote Tiam1-mediated activation of Rac *in vivo* [26]. Further research is required to elucidate other mechanisms involved in Tiam1 translocation and activation in MDR lymphoma cell lines. Although inhibition of PI3K was able to transiently decrease Tiam1 translocation to the plasma membrane, migration towards HA was significantly inhibited. This suggests that although PI3K-independent mechanisms may induce Tiam1 translocation to the plasma membrane, the PI3K-dependent mechanism of Tiam1 activation may be essential for migration of these cell lines towards HA.

In summary, we described for the first time the relationship among HA, Tiam1, migration and MDR in murine lymphoma cells. In this work, we demonstrated that MDR lymphoma cell lines presented a higher migratory capacity towards HA *in vitro* as well as a higher constitutive active Tiam1 expression than the sensitive cell line. Besides, we suggest a connection between HA/CD44 interaction and MDR in lymphoma cell lines, based on CD44 ability to preferentially induce migration towards HA of Pgp+ cells. In addition, we also demonstrated that HA treatment induces Tiam1 activation in the resistant cell lines, leading to an increase in their migration towards HA *in vitro*. Moreover, our data suggest that HA/CD44 interaction is crucial for HA induction of Tiam1 activation. Finally, inhibition of the PI3K pathway decreased migration towards HA and Tiam1 activation indicating that the PI3K pathway – which is up-regulated in the cell lines – may be essential in Tiam1 activation and migration towards HA in these MDR lymphoma cells. Our results suggest a novel role of HA in migration and MDR in lymphoma and that disruption of HA/CD44 signaling (using molecules like CD44 blocking antibodies) may be useful in the treatment of MDR hematological malignancies.

Conflicts of interest

None.

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Contributions: RCR performed most of the experiments, contributed to the design of the study, analyzed the data and wrote the manuscript. LA performed the transfection experiments and confocal microscopy, contributed to the design of the study, editing and supervision of the manuscript. NS contributed to the transfection of the cell lines. SL contributed to statistical analysis. GB and EA contributed to the design of the study. MGG contributed to the design of the study, analysis of the data and editing of the manuscript. SH is the director of the group and supervised the work. All authors read and approved the final manuscript.

References

[1] Gottesman MM, Ling V. The molecular basis of multidrug resistance in cancer: the early years of P-glycoprotein research. FEBS Lett 2006;580:998–1009.
[2] Bellacosa A, Kumar CC, Di Cristofano A, Testa JR. Activation of AKT kinases in cancer: implications for therapeutic targeting. Adv Cancer Res 2005;94: 29–86.
[3] Mashima T, Tsuruo T. Defects of the apoptotic pathway as therapeutic target against cancer. Drug Resist Updat 2005;8:339–43.
[4] Luciani F, Molinari A, Lozupone F, Calcabrini A, Lugini L, Stringaro A, et al. P-glycoprotein-actin association through ERM family proteins: a role in P-glycoprotein function in human cells of lymphoid origin. Blood 2002;99: 641–8.
[5] Li ZW, Dalton WS. Tumor microenvironment and drug resistance in hematologic malignancies. Blood Rev 2006;20:333–42.
[6] Johnson P, Ruffell B. CD44 and its role in inflammation and inflammatory diseases. Inflamm Allergy Drug Targets 2009;8:208–20.
[7] Toole BP. Hyaluronan in morphogenesis. Semin Cell Dev Biol 2001;12:79–87.
[8] Jiang D, Liang J, Noble PW. Hyaluronan in tissue injury and repair. Annu Rev Cell Dev Biol 2007;23:435–61.
[9] Toole BP. Hyaluronan: from extracellular glue to pericellular cue. Nat Rev Cancer 2004;4:528–39.
[10] Boregowda RK, Appaiah HN, Siddaiah M, Kumarswamy SB, Sunila S, Thimmaiah KN, et al. Expression of hyaluronan in human tumor progression. J Carcinog 2006;5:2.
[11] Itano N, Kimata K. Altered hyaluronan biosynthesis in cancer progression. Semin Cancer Biol 2008;18:268–74.
[12] Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol 2003;4:33–45.
[13] Naor D, Wallach-Dayan SB, Zahalka MA, Sionov RV. Involvement of CD44, a molecule with a thousand faces, in cancer dissemination. Semin Cancer Biol 2008;18:260–7.
[14] Bourguignon LY. Hyaluronan-mediated CD44 activation of RhoGTPase signaling and cytoskeleton function promotes tumor progression. Semin Cancer Biol 2008;18:251–9.
[15] Minard ME, Kim LS, Price JE, Gallick GE. The role of the guanine nucleotide exchange factor Tiam1 in cellular migration, invasion, adhesion and tumor progression. Breast Cancer Res Treat 2004;84:21–32.
[16] Habets GG, Scholtes EH, Zuydgeest D, van der Kammen RA, Stam JC, Berns A, et al. Identification of an invasion-inducing gene, Tiam1, that encodes a protein with homology to GDP-GTP exchangers for Rho-like proteins. Cell 1994;77:537–49.
[17] Mertens AE, Roovers RC, Collard JG. Regulation of Tiam1-Rac signalling. FEBS Lett 2003;546:11–6.
[18] Adam L, Vadlamudi RK, McCrea P, Kumar R. Tiam1 overexpression potentiates heregulin-induced lymphoid enhancer factor-1/beta-catenin nuclear signaling in breast cancer cells by modulating the intercellular stability. J Biol Chem 2001;276:28443–50.
[19] Lane J, Martin TA, Mansel RE, Jiang WG. The expression and prognostic value of the guanine nucleotide exchange factors (GEFs) Trio, Vav1 and TIAM-1 in human breast cancer. Int Semin Surg Oncol 2008;5:23.
[20] Minard ME, Herynk MH, Collard JG, Gallick GE. The guanine nucleotide exchange factor Tiam1 increases colon carcinoma growth at metastatic sites in an orthotopic nude mouse model. Oncogene 2005;24:2568–73.
[21] Ding Y, Chen B, Wang S, Zhao L, Chen J, Chen L, et al. Overexpression of Tiam1 in hepatocellular carcinomas predicts poor prognosis of HCC patients. Int J Cancer 2009;124:653–8.
[22] Stam JC, Michiels F, van der Kammen RA, Moolenaar WH, Collard JG. Invasion of T-lymphoma cells: cooperation between Rho family GTPases and lysophospholipid receptor signaling. EMBO J 1998;17:4066–74.
[23] Engers R, Springer E, Michiels F, Collard JG, Gabbert HE. Rac affects invasion of human renal cell carcinomas by up-regulating tissue inhibitor of metalloproteinases (TIMP)-1 and TIMP-2 expression. J Biol Chem 2001;276: 41889–97.
[24] Fleming IN, Gray A, Downes CP. Regulation of the Rac1-specific exchange factor Tiam1 involves both phosphoinositide 3-kinase-dependent and -independent components. Biochem J 2000;351:173–82.

[25] Fleming IN, Batty IH, Prescott AR, Gray A, Kular GS, Stewart H, et al. Inositol phospholipids regulate the guanine-nucleotide-exchange factor Tiam1 by facilitating its binding to the plasma membrane and regulating GDP/GTP exchange on Rac1. Biochem J 2004;382:857–65.
[26] Lambert JM, Lambert QT, Reuther GW, Malliri A, Siderovski DP, Sondek J, et al. Tiam1 mediates Ras activation of Rac by a PI(3)K-independent mechanism. Nat Cell Biol 2002;4:621–5.
[27] Misra S, Ghatak S, Zoltan-Jones A, Toole BP. Regulation of multidrug resistance in cancer cells by hyaluronan. J Biol Chem 2003;278:25285–8.
[28] Bourguignon LY, Spevak CC, Wong G, Xia W, Gilad E. Hyaluronan-CD44 interaction with PKC-epsilon promotes oncogenic signaling by the stem cell marker, Nanog and the production of microRNA-21 leading to downregulation of the tumor suppressor protein, PDCD4, anti-apoptosis and chemotherapy resistance in breast tumor cells. J Biol Chem 2009;284(39):26533–46.
[29] Ohashi R, Takahashi F, Cui R, Yoshioka M, Gu T, Sasaki S, et al. Interaction between CD44 and hyaluronate induces chemoresistance in non-small cell lung cancer cell. Cancer Lett 2007;252:225–34.
[30] Wang SJ, Bourguignon LY. Hyaluronan and the interaction between CD44 and epidermal growth factor receptor in oncogenic signaling and chemotherapy resistance in head and neck cancer. Arch Otolaryngol Head Neck Surg 2006;132:771–8.
[31] Slomiany MG, Dai L, Bomar PA, Knackstedt TJ, Kranc DA, Tolliver L, et al. Abrogating drug resistance in malignant peripheral nerve sheath tumors by disrupting hyaluronan-CD44 interactions with small hyaluronan oligosaccharides. Cancer Res 2009;69:4992–8.
[32] Cordo Russo RI, Garcia MG, Alaniz L, Blanco G, Alvarez E, Hajos SE. Hyaluronan oligosaccharides sensitize lymphoma resistant cell lines to vincristine by modulating P-glycoprotein activity and PI3K/Akt pathway. Int J Cancer 2008;122:1012–8.
[33] Bourguignon LY, Peyrollier K, Xia W, Gilad WE. Hyaluronan-CD44 interaction activates stem cell marker Nanog, Stat-3-mediated MDR1 gene expression, and ankyrin-regulated multidrug efflux in breast and ovarian tumor cells. J Biol Chem 2008;283:17635–51.
[34] Miletti-Gonzalez KE, Chen S, Muthukumaran N, Saglimbeni GN, Wu X, Yang J, et al. The CD44 receptor interacts with P-glycoprotein to promote cell migration and invasion in cancer. Cancer Res 2005;65:6660–7.
[35] Colone M, Calcabrini A, Toccacieli L, Bozzuto G, Stringaro A, Gentile M, et al. The multidrug transporter P-glycoprotein: a mediator of melanoma invasion? J Invest Dermatol 2008;128:957–71.
[36] Lopes EC, Garcia MG, Vellon L, Alvarez E, Hajos SE. Correlation between decreased apoptosis and multidrug resistance (MDR) in murine leukemic T cell lines. Leuk Lymphoma 2001;42:775–87.
[37] Alaniz L, Garcia MG, Gallo-Rodriguez C, Agusti R, Sterin-Speziale N, Hajos SE, et al. Hyaluronan oligosaccharides induce cell death through PI3-K/Akt pathway independently of NF-kappaB transcription factor. Glycobiology 2006;16:359–67.
[38] Staroselsky AN, Mahlin T, Savion N, Klein O, Nordenberg J, Donin N, et al. Metastatic potential and multidrug resistance correlation in the B16 melanoma system. J Exp Ther Oncol 1996;1:251–9.
[39] Bjornland K, Lehne G, Johansen HT, Fodstad O, Rugstad HE, Aasen AO, et al. Human hepatoma cells rich in P-glycoprotein display enhanced in vitro invasive properties compared to P-glycoprotein-poor hepatoma cells. Oncol Res 1998;10:255–62.
[40] Yang JM, Vassil A, Hait WN. Involvement of phosphatidylinositol-3-kinase in membrane ruffling induced by P-glycoprotein substrates in multidrug-resistant carcinoma cells. Biochem Pharmacol 2002;63:959–66.
[41] Bacso Z, Nagy H, Goda K, Bene L, Fenyvesi F, Matko J, et al. Raft and cytoskeleton associations of an ABC transporter: P-glycoprotein. Cytometry A 2004;61:105–16.
[42] Lopes EC, Ernst G, Aulicino P, Vanzulli S, Garcia M, Alvarez E, et al. Dissimilar invasive and metastatic behavior of vincristine and doxorubicin-resistant cell lines derived from a murine T cell lymphoid leukemia. Clin Exp Metastasis 2002;19:283–90.
[43] Lesley J, Hascall VC, Tammi M, Hyman R. Hyaluronan binding by cell surface CD44. J Biol Chem 2000;275:26967–75.
[44] Gál I, Lesley J, Ko W, Gonda A, Stoop R, Hyman R, et al. Role of the extracellular and cytoplasmic domains of CD44 in the rolling interaction of lymphoid cells with hyaluronan under physiologic flow. J Biol Chem 2003;278:11150–8.
[45] Goldberg RL, Seidman JD, Chi-Rosso G, Toole BP. Endogenous hyaluronante-cell surface interactions in 3T3 and simian virus-transformed 3T3 cells. J Biol Chem 1984;259:9440–6.
[46] Toole BP. Hyaluronan-CD44 interactions in cancer: paradoxes and possibilities. Clin Cancer Res 2009;15(24):7462–8.
[47] Bourguignon LY, Zhu H, Shao L, Chen YW. CD44 interaction with tiam1 promotes Rac1 signaling and hyaluronic acid-mediated breast tumor cell migration. J Biol Chem 2000;275:1829–38.
[48] Garcia MG, Alaniz LD, Cordo Russo RI, Alvarez E, Hajos SE. PI3K/Akt inhibition modulates multidrug resistance and activates NF-kappaB in murine lymphoma cell lines. Leuk Res 2009;33:288–96.
[49] Teranishi F, Takahashi N, Gao N, Akamo Y, Takeyama H, Manabe T, et al. Phosphoinositide 3-kinase inhibitor (wortmannin) inhibits pancreatic cancer cell motility and migration induced by hyaluronan in vitro and peritoneal metastasis in vivo. Cancer Sci 2009;100:770–7.
[50] Strumane K, Song JY, Baas I, Collard JG. Increased Rac activity is required for the progression of T-lymphomas induced by Pten-deficiency. Leuk Res 2009;32:113–20.