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Human leukaemic cell lines synthesize hyaluronan to avoid senescence and resist chemotherapy

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Abstract

Hyaluronan is one of the major components of the extracellular matrix. Several solid tumours produce high levels of hyaluronan, which promotes survival and multidrug resistance. Hyaluronan oligomers can block hyaluronan effects. However, little is known about the role of hyaluronan in haematological malignancies. The aim of this work was to determine whether hyaluronan or its oligomers can modulate the proliferation of leukaemia cells as well as their effect on multidrug resistance. Receptors and signalling pathways involved were also analysed. For this purpose the human leukaemic cell lines K562 and Kv562, which are sensitive and resistant to Vincristine, respectively, were used. We demonstrated that hyaluronan induced cell proliferation in both cell lines. On K562 cells, this effect was mediated by CD44 and activation of both PI3K/Akt and MEK/ERK pathways, whereas on Kv562 cells the effect was mediated by RHAMM and PI3K/Akt activation. The inhibition of hyaluronan synthesis by 4-Methylumbelliferone decreased cell lines proliferation and sensitized Kv562 to the effect of Vincristine through Pgp inhibition, in both cases with senescence induction. Moreover, hyaluronan oligomers inhibited K562 proliferation mediated by CD44 as well as Akt and ERK downregulation. Furthermore, hyaluronan oligomers sensitized Kv562 cells to Vincristine by Pgp inhibition inducing senescence. We postulate that the synthesis of hyaluronan would promote leukaemia progression mediated by the triggering of the above mentioned proliferative signals. These findings highlight the potential use of hyaluronan oligomers and 4-Methylumbelliferone as coadjuvant for drug-resistant leukaemia.

Keywords: Hyaluronan / CD44 / RHAMM / Senescence / Drug-resistance / Leukaemia.

Introduction

Tumour progression is accomplished by several mechanisms such as extracellular matrix (ECM) modification, among others. It is known that the microenvironment can enhance cell proliferation, migration and multidrug resistance (MDR). In different types of solid tumours, many authors have reported the presence of increased levels of hyaluronan (HA), one of the major ECM components, which has been associated with worse prognosis (Auvinen et al. 2013; Boregowda et al. 2006; Itano et al. 2008; Provenzano and Hingorani 2013). Nevertheless, little is known about the role of HA in oncohaematological processes.

HA is a linear glycosaminoglycan (GAG), composed of repeated disaccharide units of [D-glucuronic acid β (1→3) and N-acetyl-D-glucosamine β (1→4)]n. Its molecular weight ranges from 10^5 to 10^7 Da (Csoka and Stern 2013). HA levels result from a balance between its synthesis mediated by hyaluronan synthases (HAS1-3), its internalization by receptors and its degradation by hyaluronidases (HYAL1-3) (Jiang D et al. 2011; Toole 2004). Apart from its physicochemical role, HA plays a role by interacting with cells to promote adhesion and motility, proliferation and differentiation (Toole 2009). It is known that a high amount of HA is present at the haematopoietic stem cell niche where it confers genomic protection through its antioxidant properties and by activating P-glycoprotein (Pgp) which extrudes genotoxic agents (Darzynkiewicz and Balazs 2012). At the cellular level, all these mechanisms are triggered after the interaction of HA with its receptors. CD44 is the main HA receptor, however, others such as RHAMM, HARE, Lyve-1, TLR-2 and TLR-4 have been described (Jiang D et al. 2011; Toole 2004; Toole 2009).

CD44 is a single chain transmembrane glycoprotein encoded by a gene located on chromosome 11 short arm. This molecule occurs as several isoforms generated by alternative splicing of variant exons (exon 6 to 15)(Wang and Bourguignon 2011). CD44 has a critical role in cell proliferation, differentiation, migration and adhesion (Johnson and Ruffell 2009). After cross linking of HA with CD44 the intracytoplasmatic domain promotes activation of several signalling pathways such as PI3K/Akt and MAPK (Naor et al. 2008; Toole 2009; Wang and Bourguignon 2011).

RHAMM has been described as an itinerant protein found at the membrane surface but also in the cytoplasm and nucleus of the cell (Maxwell et al. 2008; Telmer et al. 2011). It is known that HA-RHAMM interaction results in binding between RHAMM and transmembrane proteins such as CD44 or tyrosine kinase receptors thus leading to the activation of PI3K/Akt and MEK/ERK signalling pathways (Goueffic et al. 2006; Maxwell et al. 2008; Telmer et al. 2011).

Several types of solid tumours overexpress CD44 and RHAMM and synthesize large amounts of HA promoting tumour progression. HA interacts with its receptors and triggers different signalling pathways such as PI3K/Akt and MAPK, which are involved in cell proliferation, MDR and migration (Toole 2004; Toole 2009). Besides, the HA-CD44 interaction stimulates Pgp activity and promotes drug extrusion thus enhancing MDR (Bourguignon et al. 2008). In this way, some alternatives have been postulated to attenuate the signals triggered by HA, as the use of HA oligomers (oHA) (which are short molecules that cannot crosslink receptors, thus blocking the HA effects), antibodies against receptors or soluble HA binding protein. All these variants have been studied mainly in solid tumours (Toole 2004; Toole 2009). The implication of HA in the pathogenesis of chronic myeloid leukaemia (CML) as well as the alternatives to block its effects have been poorly studied so far.

CML is a myeloproliferative syndrome characterized by the presence of the Philadelphia chromosome (Ph), generated by a reciprocal translocation occurring between chromosomes 9 and 22 [t(9;22)(q34;q11)] (Nowell and Hungerford 1960; Rowley 1973). As a consequence, a fusion gene (*bcr-abl*) encoding a constitutively active kinase is generated. The first line treatment consists on *BCR-ABL* inhibitors such as Imatinib, Nilotinib and Dasatinib (Bubnoff and Duyster 2010). Nevertheless when such drugs are not effective, conventional chemotherapy with Vincristine or Doxorubicin is used. Recent studies have demonstrated that both Imatinib and Doxorubicin are able to induce senescence on K562, a cell line model for CML (Drullion et al. 2012; Yang et al. 2012).

Senescence is a terminal differentiation stage characterized by molecular and morphological changes of the cell, associated with ageing, stress conditions and tumour suppression. It is known that senescent cells are featured by: a) an irreversible cell cycle arrest with overexpression of cell cycle inhibitors such as p53 and p21, b) an increase in cellular granulation and size, c) the presence of high β-galactosidase activity at suboptimal pH (SA-β-gal), d) resistance to apoptosis, e) changes in heterochromatin architecture called senescence-associated heterochromatin foci (SAHF) (Campisi and d'Adda di Fagagna 2007; Rodier and Campisi 2011; Yang et al. 2012). Besides, recent studies have shown that the K562 CML cell line is homozygous p53 and p16 negative (Yang et al. 2012).

Due to the scarce literature data regarding the implication of HA in oncohaematological processes, the aim of this work was to study some biological mechanisms stimulated by HA. As a human CML model the K562 and Kv562 cell lines were employed. These cells are sensitive and resistant to Vincristine (VCR), respectively. Therefore, the ability of HA or oHA to modulate leukaemia cells proliferation and their effects on MDR were assessed. The receptors

and signalling pathways involved in the process were also analysed. Our results showed that HA treatment stimulated the proliferation of both cell lines. On K562 cells, this effect was mediated by CD44 as well as PI3K/Akt and MEK/ERK pathways while on Kv562, the effect was mediated by RHAMM and PI3K/Akt. Moreover, oHA and VCR inhibited K562 cell proliferation, however, these agents failed to modify Kv562 cell growth. The HA synthesis inhibitor, 4-Methylumbelliferone (4MU), decreased the proliferation of both cell lines by decreasing the pAkt/Akt ratio and inducing senescence. The co-treatment of cells with 4MU and HA abrogated such effects. Both oHA and 4MU were able to sensitize Kv562 to VCR and to inhibit Pgp. Moreover, oHA and 4MU in combination with VCR induced senescence. To our knowledge, this is the first work reporting a direct effect of HA favouring escape of senescence.

Results

Characterization of K562 and Kv562 cell lines

To characterize the cell lines, HA secretion and CD44 and RHAMM expression were evaluated as well as the HA-CD44 and HA-RHAMM interactions. The amounts of HA secreted by cells after 72 h of culture were 480±53 ng/ml (625±15 ng/10^6 cells) and 448±51 ng/ml (620±10 ng/10^6 cells) for K562 and Kv562 cell lines, respectively. Treatment with 500 µM and 100 µM 4MU resulted in a reduction of HA of 82% and 72% in K562 cells and 84% and 71% in Kv562 cells, respectively (Figure 1A). Expression of CD44 and RHAMM were evaluated by indirect immunoflourescence (IIF), flow cytometry (FC) and western blot (WB). IIF demonstrates that both cell lines expressed CD44 and RHAMM (Figure1B, panel I). Panel II shows CD44 and RHAMM expression on membrane surface evaluated by FC while panel III confirms the expression of both receptors evaluated by WB. It is important to note that IFF shows total

RHAMM expression since cells were permeabilized (Figure 1B panel I) while FC shows only surface membrane expression (Figure 1B, panel II). Figure 1C shows that both cell lines were able to bind HA (82±3 % in the case of K562 and 80±1 % in the case of Kv562) and such binding was abrogated by pre-treatment with HA, oHA, anti-CD44 Km81 or anti-RHAMM Ab.

HA increases cell proliferation through CD44 in K562 and RHAMM in Kv562.

Since cells proved to express CD44 and RHAMM, the effect of HA and oHA treatment on cell growth was assessed. To evaluate whether HA, oHA or VCR modulate cell proliferation, the [^{3}H]-thymidine uptake was measured. Signalling pathways and receptors involved were also analysed. Several concentrations (50, 100, 200, 300 and 500 µg/ml) of HA and oHA were tested. 300 µg/ml of HA or oHA were used since it was the lower dose which modulated cell proliferation (data not shown). Figure 2A shows that HA increased cell growth by 19±3% and 33±8% in K562 and Kv562 cells. In K562 cells, VCR completely abrogated cell growth while oHA decreased cell proliferation by 18±2%. Contrarily oHA and VCR did not modify Kv562 proliferation. Figures 2B and 2C show that in K562 cells, the effect of HA was reverted by anti-CD44 Km81, LY294002 or UO126 co-incubation suggesting that CD44 as well as PI3K and MEK signalling pathways were necessary for the HA effect. However, the HA-induced proliferation on Kv562 was abolished by anti-RHAMM Ab and LY294002, suggesting that HA would exert its effect through RHAMM and PI3K. Figure 2B also shows that the anti-proliferative effect of oHA was mediated by CD44 in K562 cells. Controls with anti-CD44 Km81, anti-RHAMM Ab, Ly294002 and UO126 had no effect on cell growth.

HA modulates PI3K/Akt and MEK/ERK signalling pathways

To confirm that HA stimulates PI3K/Akt and MEK/ERK signalling pathways as well as the receptors involved, a WB assay was performed. Figure 3A shows that HA induced an increase of 90±10 % for pAkt/Akt and 50±5 % for pERK/ERK ratios on K562 cells while oHA decreased such ratios to 65±5% and 68±10 % respectively. However, on Kv562 cells, HA increased only the pAkt/Akt ratio (211±46%) while oHA decreased it (61±8 %). Figure 3B shows that PI3K inhibition with Ly294002 decreased the HA-induced Akt phosphorylation on both cell lines. The inhibition of MEK by UO126 decreased not only ERK but also Akt phosphorylation, suggesting a cross-talk between both signalling pathways on K562 cells. Contrarily, the inhibition of MEK did not affect Akt phosphorylation on Kv562. Figure 3C shows that HA failed to exert its effects in the presence of anti-CD44 Km81 on K562 but not on Kv562. Instead, on these cells the effect was mediated by RHAMM (Figure 3D). Treatment with anti-CD44 Km81 or anti-RHAMM Ab did not modify either pAkt/Akt or pERK/ERK ratios.

The inhibition of HA synthesis decreased cell proliferation

As both cell lines secreted HA which increased cell proliferation, it was interesting to investigate the role of HA produced by cells themselves. The HA synthesis was inhibited and cell proliferation as well as Akt and ERK phosphorylation were evaluated. Figure 4A shows that 500 μM and 100 μM 4MU inhibited both cell lines proliferation. Co-treatment with 500 μM 4MU + 300 μg/ml HA partially reverted this effect while co-treatment with 100 μM 4MU + 300 μg/ml HA restored baseline conditions. Moreover, cotreatment with 100 μM 4MU + 300 ng/ml HA also restored basal proliferation of both cell lines. Therefore, the inhibition of HA synthesis with low concentrations of 4MU decreased cell proliferation while the addition of minor amounts of HA restored cell growth suggesting an autocrine or paracrine effect. Besides, high concentrations of 4MU would be able to trigger anti-proliferative signals by itself. In K562 cells, the effect of

4MU+HA on cell proliferation was abolished by blocking CD44. On the contrary, in Kv562 this effect was mediated by blocking RHAMM, suggesting that cell proliferation induced by HA in K562 and Kv562 would be mediated by CD44 and RHAMM, respectively. Figure 4B shows that 4MU decreased pAkt/Akt and pERK/ERK ratios on K562 and pAkt/Akt ratio on Kv562. These effects were abrogated by 4MU+HA co-treatment. Therefore, both cell lines secrete HA which is able to trigger proliferative signals.

The inhibition of HA synthesis induces senescence

Since 4MU inhibited both cell lines proliferation, cell death was evaluated by FC demonstrating that 4MU failed to induce cell death (Figure 5A). Therefore, we analysed senescence induction. Figure 5B panel I shows that 500μM as well as 100μM 4MU increased SA-β-gal activity in both cell lines while the addition of HA reverted 4MU effect. Treatment with 1 μM VCR enhanced SA-β-gal activity only in K562 cells but not in the resistant cell line. Panel II shows that 4MU induced the presence of SAHF in both cell lines while the addition of HA restored the baseline conditions. Panel III shows that 4MU was able to arrest cell cycle in G2/M while the addition of HA reverted 4MU effect. From this result, we suggest that HA is able to trigger anti-senescence signals.

4MU sensitizes Kv562 cell line to the effect of VCR through Pgp inhibition and senescence induction

Taking into account that the interaction HA-CD44 triggers the activation of Pgp (Bourguignon et al. 2008) and that Kv562 expresses CD44 and Pgp (Cordo Russo et al. 2008), it was interesting to analyse whether the inhibition of HA synthesis could decrease Pgp activity and sensitize cells to the effect of VCR. For this purpose, only the VCR-resistant Kv562 cells were used. Figure 6A shows that a significant reduction of Kv562 growth occurred upon treatment of

cells with both 4MU concentrations (500 μM and 100 μM) plus VCR (1 μM). These effects were reverted by co-incubation of 4MU+VCR with HA. Therefore, the effect of 4MU on Pgp functionality was studied. Figure 6B shows that the Pgp inhibitor cyclosporine A (CsA) was able to block the drug efflux, thus confirming the presence of a functional pump on Kv562 cells. It was observed that 500 μM and 100 μM 4MU increased Doxorubicin accumulation by a 37±9 % and 28±2 %, respectively. Co-incubation of cells with 4MU+HA reverted the effect of 4MU whereas co-treatment plus anti-CD44 Km81 restored the effect. No changes in Doxorubicin efflux were observed with anti-CD44 Km81 alone. Since 4MU+VCR inhibited Kv562 cell proliferation and 4MU induced cell senescence, the latter mechanism was assessed. Figure 6C shows that co-treatment with 4MU+VCR also increased SA-β-gal activity (panel I), induced SAHF (panel II) and augmented cell cycle arrest in G2/M (panel III). The latter effects were abrogated by co-incubation of 4MU+VCR with HA. Therefore the synthesis of HA would be crucial to resist to chemotherapy.

oHA sensitizes Kv562 cell line to the effect of VCR through Pgp inhibition and the induction of senescence

Since the inhibition of HA synthesis rendered Kv562 cells sensitive to the effect of VCR and knowing that oHA was able to displace HA binding, the role of oHA on MDR was analysed. To this end, cell proliferation, Pgp activity and the induction of senescence were evaluated. The co-treatment with oHA+VCR decreased Kv562 cells proliferation by a 32±2 %, which was recovered by co-incubation of cells with oHA+VCR and anti-CD44 Km81 (Figure 7A). Pgp activity was evaluated by the intracellular accumulation of Doxorubicin showing that oHA induced an increase of 35±3 % of such drug. To evaluate whether the effect of oHA on Pgp function was mediated by interaction with CD44, resistant cells were treated with anti-CD44

Km81 plus oHA. The blocking effect of oHA on Pgp efflux was abolished indicating that oHA could modulate Pgp activity through CD44. No changes in Doxorubicin efflux were observed with anti-CD44 Km81 alone (Figure 7B). To confirm that oHA sensitized Kv562 and inhibited Pgp through downregulation of Akt, cells were incubated with 5 μM Ly294002 (a PI3K inhibitor) showing that Ly294002 completely sensitized Kv562 to the effect of VCR without modifying Pgp activity (Figures 7A and B). UO126 (MEK inhibitor) failed to sensitize Kv562 (Figure 7A). Finally, cell senescence was analysed. The co-treatment with oHA+VCR increased SA-β-gal activity, induced SAHF presence as well as arrested the cell cycle in G2/M (Figure 7C panel I, II and III, respectively). Therefore, this co-treatment enhanced senescence induction sensitizing Kv562 cells to the effect of VCR through Pgp and Akt inhibition.

Discussion

CML represents the 15% of all leukaemias, with an incidence of 2 cases/100000 people per year. In 2012, about 140000 people worldwide were diagnosed with CML. Although Imatinib is a highly efficient drug, after some time, resistant cells may be selected leading to therapeutic failure. Current studies on CML biology are vital to find new molecular targets to improve current therapies.

In this work we demonstrated that HA triggers several proliferative signalling pathways which promoted cell proliferation and MDR while avoiding senescence (Figure 8). It is well known that haematopoietic stem cell niches have high amounts of HA which is crucial for homing during bone marrow transplantation (Darzynkiewicz and Balazs 2012). We hypothesize that HA would play a crucial role in stem cells since it triggers signalling pathways that lead to the abrogation of senescence and avoid ageing. However, when the HA concentration increases above

physiological levels, it may confer a positive feedback on cell proliferation and Pgp activity leading to leukaemia progression.

We demonstrated that K562 and Kv562 secreted significant amounts of HA and that 4MU was able to inhibit its synthesis. Both cell lines expressed CD44 and RHAMM. These results were in agreement with other authors who have demonstrated a significant expression of RHAMM on K562 cells (Greiner et al. 2006; Schmitt et al. 2009).

We also showed that on K562 cells, HA increased cell proliferation while oHA decreased it. We hypothesize that 300 μg/ml oHA would be enough to displace endogenous HA while lower doses may be unable. The HA-CD44 interaction activated PI3K/Akt and MEK/ERK while the oHA-CD44 interaction partially abrogated these signalling pathways, in agreement with previous reports on other tumour cell lines (Toole 2009). After treatment with UO126, a decrease on Akt phosphorylation was observed, suggesting that MEK or other downstream proteins could be phosphorylating Akt. Our results are in agreement with previous studies demonstrating a crosstalk between both signalling pathways in several cancer cells such as pheochromocytoma (Yang et al. 2011) and hepatocellular carcinoma cell lines (Dai et al. 2009). Besides, HA treatment increased Akt phosphorylation mediated by PI3K and MEK pathways while increasing the pERK/ERK ratio mediated by MEK. HA also increased cell growth on the resistant cell line Kv562, in this case the effect was mediated by RHAMM. Moreover, it has been reported the activation of PI3K mediated by HA-RHAMM interaction on vascular smooth muscle cell (Goueffic et al. 2006). As described previously, RHAMM is not a transmembrane receptor; therefore, HA-RHAMM interaction activates other cell surface receptors such as CD44 or PDGFR to exert the effect (Maxwell et al. 2008; Telmer et al. 2011). The HA-RHAMM interaction might stimulate a transmembrane receptor leading to the activation of PI3K/Akt but

not to MEK/ERK. It is noteworthy that the HA-induced Kv562 cells growth required the PI3K/Akt pathway, therefore on the resistant cell line this signalling pathway was more important than MEK/ERK.

In this work, we demonstrated that 4-MU is an effective anti-proliferative agent. This result is in agreement with other authors who have demonstrated that 4MU can inhibit cell proliferation in several tumour cells such as osteosarcoma cells, oesophageal squamous cells carcinoma, breast cancer cells and hepatocellular carcinoma (Arai et al. 2011; Piccioni et al. 2011; Twarock et al. 2011; Urakawa et al. 2012). We also demonstrated that co-treatment with HA reverted the 4MU anti-proliferative effect. On K562 cells the effect of HA was mediated by CD44 while on Kv562 it was mediated by RHAMM. Therefore, the HA synthesized by cells themselves would have an autocrine or paracrine effect which is important for the growth of both cell lines. The latter finding is in line with other reports demonstrating similar results after 4-MU+HA co-treatment on prostate cancer cells (Lokeshwar et al. 2010). Moreover, we showed that on K562, 4MU decreased pAkt/Akt and pERK/ERK ratios while on Kv562 only Akt was down-regulated. These effects were reverted by co-treatment with HA. We suggest that the HA synthesized by cells themselves would be activating such signalling pathways. All these results correlate with the effect of HA treatment described above.

Furthermore, we described for the first time that 4MU induced cellular senescence while the co-treatment with HA abrogated this effect. It is well known that cellular senescence is an important tumour suppressor mechanism (Campisi and d'Adda di Fagagna 2007; Rodier and Campisi 2011). In this sense, HA would be leading to leukaemia progression. We also suggest a potential relationship between the PI3K/Akt signalling pathway and senescence. It is known that Akt is crucial to cell cycle progression (Chang et al. 2003) and that senescence is characterized by cell

cycle arrest (Campisi and d'Adda di Fagagna 2007; Drullion et al. 2012; Rodier and Campisi 2011; Yang et al. 2012). Our results demonstrated that HA activated Akt, which would be crucial to avoid cellular senescence, and induced cell cycle progression on K562 and Kv562 cells. Several authors have reported a pro-apoptotic effect of 4MU on different tumour cells (Arai et al. 2011; Lokeshwar et al. 2010; Piccioni et al. 2011; Urakawa et al. 2012), however we did not observe cell death upon treatment with this inhibitor. Moreover, VCR treatment on K562 completely inhibited cell growth without inducing cell death. It should be noted that the lethal dose 50 was 250μM for K562 and 600μM for Kv562, showing a significant death resistance (data not shown).

MDR in haematological malignancies is the main reason of chemotherapy failure. Since multiple factors may contribute to chemoresistance, the impact of extracellular matrix components on this process has become of great interest (Baumgartner et al. 1998; Bissell and Radisky 2001; Cordo Russo et al. 2008; Croix et al. 1998). Thus, we evaluated the role of HA on MDR. The inhibition of HA synthesis with 4MU sensitized Kv562 cells to the effect of VCR. Co-treatment with 4MU and VCR plus HA reverted this effect. Furthermore, 4MU inhibited the efflux of Doxorubicin while co-treatment with HA restored Pgp activity, by a process mediated by CD44. Similar results were observed by Bourguignon LY et al, 2008 who have reported that HA-CD44 interaction induces ankyrin activation increasing Pgp activity in breast and ovarian cancer cells (Bourguignon et al. 2008). We also showed that 4MU+VCR induced senescence and that co-treatment plus HA abrogated it, suggesting that HA would be important not only to avoid senescence but also to resist chemotherapy activating drug efflux.

Since we have reported that oHA are able to inhibit Pgp activity (Cordo Russo et al. 2008) we decided to study the role of oHA on MDR. Similar results were obtained either for 4MU or oHA.

The latter was able to sensitize Kv562 cells to the effect of VCR inhibiting Pgp through CD44. These results are in agreement with Xiangzhen Cui et al, 2009 who demonstrated that oHA can sensitize K562/A02 cells (Adryamicin resistant) and with Slomiany MG, 2009 et al who demonstrated that oHA sensitized malignant peripheral nerve sheath tumours to apoptotic death (Cui et al. 2009; Slomiany et al. 2009). We also reported for the first time that oHA+VCR induced cellular senescence in Kv562 leukaemia cells.

The PI3K/Akt signalling pathway enhances MDR through its anti-apoptotic effect and its ability to activate Pgp (McCubrey et al. 2007 ; Rosen et al. 2013). Besides, previous works carried out in our laboratory have shown a direct effect of PI3K/Akt signalling pathway on Pgp activity in murine lymphoma cell lines (García MG et al. 2009). We were able to sensitize Kv562 cells to the effect of VCR when the PI3K pathway was inhibited. However, Ly294002 did not inhibit Pgp activity suggesting that Kv562 cells become VCR resistant not only by Pgp efflux but also through the PI3K/Akt activity. It should be noted that 4MU and oHA downregulated this signalling pathway, therefore, we postulate that 4MU and oHA would sensitize Kv562 cells not only by inhibiting Pgp but also by decreasing Akt phosphorylation.

The induction of senescence would be a suitable alternative for leukaemia treatment since it would allow the development of normal haematopoietic cells while preventing the proliferation of tumour cells mediated by a cell cycle arrest. It is important to highlight that 4MU not only is a HA synthesis inhibitor but also a non-toxic dietary supplement (Piccioni et al. 2011). Moreover, HA did not revert completely the effect observed by the highest dose tested of 4MU, suggesting that 4MU could trigger anti-proliferative signals by itself. Thus 4MU would be a potential new drug for cancer therapy.

In this work we showed for the first time that human leukaemic cell lines synthesize HA to avoid senescence and resist chemotherapy. We also postulated two ways of abrogating the effect of HA. Further studies carried out on the pathophisiology of leukaemia and the role of HA may help improve current therapies. These findings highlight the potential use of oHA and 4MU as a coadjuvant for resistant leukaemia treatment.

Materials and Methods

Cell culture

Human CML cell lines K562 (VCR sensitive) and Kv562 (VCR resistant) were grown in suspension cultures at 37ºC in a 5% CO_2 atmosphere with RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol, 100 µg/ml streptomycin and 100 IU/ml penicillin (RPMI-C). The resistant cell line was cultured in the presence of 150 ng/ml (162 nM) VCR (Cordo Russo et al. 2008). Before using, cells were thawed and subjected to three consecutive passages.

Reagents

Recombinant high molecular weight (HMW, $1.5–1.8\times10^{6}$ Da) HA (CPN spol.s.r.o Czech Republic) was supplied by Farmatrade (Argentina). Anti-CD44 monoclonal antibody (MAb) (Km81) and Alexafluor-647-conjugated HA was kindly provided by Dr. Pauline Johnson (University of British Columbia, Vancouver, Canada). Hyaluronidase bovine testes (#H3884, Type IV-S, lyophilized powder, essentially salt-free), X-gal, UO126 and LY294002 were purchased from Sigma-Aldrich USA. Biotinylated HA binding protein (bHABP, #385911) was purchased from Calbiochem USA. VCR was kindly provided by Filaxis Pharmaceuticals S.A. Argentina. Antibodies (Ab) against pAkt, Akt, pERK, ERK, β-Actin, RHAMM, anti-rat secondary horseradish peroxidase, anti-rabbit secondary horseradish peroxidase, anti-goat

secondary horseradish peroxidase, bioltinylated anti-rat secondary and biotinylated anti-goat secondary Abs were purchased from Santa Cruz Biotechnology (USA). Avidin-Phycoerythine (Av-PE) was purchased from e-Bioscience (USA). [^{3}H]-thymidine was purchased from Perkin-Elmer (Boston, USA). RPMI 1640, L-glutamine, streptomycin and penicillin were purchased from Invitrogen (Argentina).

Preparation of oHA

Oligomers ranging from HA4 tetrasaccharides to HA14 oligosaccharides were generated as described previously (Cordo Russo et al. 2008). Briefly, oligosaccharides were obtained after enzymatic digestion of LMW-HA (5 mg/ml) with Hyaluronidase bovine testes employing 500 U/mg of HA, incubating at 37ºC for 24 h. The reaction was stopped by boiling for 5 min. The suspension was then filtered through 0.22 µm filter and then Hase and oHA were separated using Centricon™ (USA) of 10 kDa pore size. oHA size was determined by HPAEC-PAD.

Measurement of HA levels by enzyme-linked immunosorbent assay (ELISA-like)

A suspension containing 5x10^5 cells was grown for 72h as described for cell culture. HA levels in cell supernatant were measured by a competitive ELISA as described previously (Cordo Russo et al. 2012). Briefly, 96 well microtiter plates were coated with 100 µg/ml HMW-HA at 4ºC. Samples and standard HMW-HA were incubated with 0.75 µg/ml bHABP at 37ºC. The plate was blocked and incubated with the samples at 37ºC for 4h. The bHABP bound was determined using an avidin–biotin detection system. Sample concentrations were calculated from a standard curve.

CD44 and RHAMM Flow Cytometry

Cells were blocked with PBS containing 2% normal human serum for 30 min at 4ºC. After incubation, anti-CD44 Km81 or anti-RHAMM Ab were added, followed by the addition of a biotinylated secondary Ab and Av-PE. All incubations were carried out for 1 h at 4ºC in the dark and with washes with cold PBS in between. Stained cells were acquired on a Pas III flow cytometer (Partec, Germany) and analysed with WinMDI 2.8 software (Scripps Institute, La Jolla, USA) (Cordo Russo et al. 2012). The corresponding isotypic controls were included in each assay.

CD44 and RHAMM Indirect Immunofluorescence

For CD44 IIF, cells were blocked with PBS containing 2% normal human serum and incubated with anti-CD44 Km81, followed by a biotinylated secondary antibody and Av-PE. Cells were then fixed with PBS plus 2% paraformaldehyde (PFA) for 15 min, washed and stained with 1 μg/ml DAPI in PBS containing 0.2% Triton X-100. For RHAMM IIF cells were blocked with PBS containing 2% normal human serum, fixed with PBS plus 2% PFA for 15 min, washed and permeabilized with PBS plus 0.2% Triton X-100 for 30 min. Cells were then incubated with anti-RHAMM Ab, followed by a biotinylated secondary antibody, and Av-PE and DAPI. In both cases, cells were analysed by an Olympus BX51 (American Inc) fluorescence microscopy (Cordo-Russo et al. 2010).

HA binding assay

Cells were incubated with PBS, 300 μg/ml HA or oHA, 10 μg/ml anti-CD44 Km81 or 5 μg/ml anti-RHAMM Ab for 1 h at 4ºC. Then, Alexafluor 647- conjugated HA was added and incubated

for 1 h. HA binding was analysed by FC on a Pas III flow cytometer (Partec, Germany). Data were analysed with WinMDI 2.8 software (Scripps Institute, La Jolla, USA).

Cell proliferation

Cell proliferation was analysed by the [^{3}H]-thymidine uptake assay evaluated at 48-72 h in 96 wells microtitre plates (Cavaliere et al. 2009). Fifty thousand cells per well (250000 cells/ml) were used. Cells were grown at 37ºC in a 5% CO2 atmosphere with RPMI-C, 300 μg/ml HA or oHA, 1 μM VCR, 500 μM 4MU, 100 μM 4MU, 10 μg/ml anti-CD44 Km81, 2 μg/ml anti-RHAMM, 5 μM Ly294002, 1 μM UO126 or combinations. After pulsing with 1μCi [^{3}H]-thymidine for 6h cells were harvested and counted in a liquid scintillation counter (Beckman, MD). Results were calculated from the mean cpm of [^{3}H]-thymidine incorporated in triplicate cultures. Untreated cells represented 100% cell survival. Cell viability at the beginning of the experiment was higher than 95%, as assessed by Trypan blue dye.

Western blot

Cells were treated with RPMI-C, 300 μg/ml HA or oHA, 500 μM 4MU, 100 μM 4MU, 10 μg/ml anti-CD44 Km81, 2 μg/ml anti-RHAMM Ab, 5 μM Ly294002, 1 μM UO126 or combinations for 24 h at 37ºC in a 5% CO_2 atmosphere. Cells were then lysed with hypotonic buffer. After centrifugation, equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Osmonics Inc, Gloucester, MA). The membrane was blocked and incubated with specific antibodies to CD44, RHAMM, p-Akt, Akt, p-ERK, ERK or β-Actin overnight at 4ºC followed by incubation with horseradish peroxidase-labelled secondary antibody for 2 h at 37ºC. The reaction was 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) (Cordo Russo et al. 2008).

Evaluation of cell viability

Cell viability was evaluated using Fluorescein diacetate (FDA) and propidium iodide (PI) by FC. Briefly, cells were treated with 4MU for 48h. Next, cells were stained with FDA for 20min, washed and incubated with PI for 5min. A Pas III flow cytometer (Partec, Germany) was used to acquire data which were analysed using WinMDI 2.8 (Scripps Institute, La Jolla, USA).

Evaluation of senescence

Cell senescence was analysed by SA-β-gal, SAHF and cell cycle (Debacq-Chainiaux et al. 2009). For SA-β-gal cells were incubated with RPMI-C, 300 µg/ml HA or oHA, 1 µM VCR, 500 µM 4MU, 100 µM 4MU or combinations at 37ºC in a 5%CO_2 atmosphere. After 48h cells were fixed with PBS plus 2% PFA and washed with PBS. Each vial was incubated for 24 h at 37ºC with 1 ml of staining solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, 150 mM NaCl, 30 mM citric acid/phosphate pH=6). Cells were washed twice with PBS and SA-β-gal activity was evaluated using an Olympus BX51 (America Inc) microscope. Blue cells were considered positive. For each condition 200 cells were counted and the percentage of SA-β-gal positive cells was calculated (Debacq-Chainiaux et al. 2009; Drullion et al. 2012). For SAHF and cell cycle evaluation, cells were subjected to similar procedure. After 48 h, cells were fixed, washed and incubated with 1 µg/ml DAPI in PBS plus 0.2% Triton X-100 for 30 min at room temperature. Cells were analysed by fluorescence microscopy (Olympus BX51, America Inc) for SAHF. For the evaluation of the cell cycle, FC was employed. A Pas III flow cytometer (Partec, Germany) was used. Acquired data were

analysed using WinMDI 2.8 (Scripps Institute, La Jolla, USA) and Cylchred 1.0.2 software (Cardiff University, United Kingdom) (Cavaliere et al. 2009; Yang et al. 2012).

Pgp activity

Intracellular accumulation of anthracyclines was carried out as previously described (Cordo Russo et al. 2008). Briefly, cells (5 x 10^5) were grown in drug-free medium for 24 h prior to analysis. Cells were then treated with RPMI-C, 8 μM Cyclosporine A (CsA), 300 μg/ml HA or oHA, 500 μM 4MU, 100 μM 4MU, 10 μg/ml anti-CD44 Km81, 5μM Ly294002, 1μM UO126 or combinations for 24 h. Then cells were incubated with 40 μM Doxorubicin for 40 min at 37ºC in a 5% CO_2 atmosphere. Stained cells were acquired and analysed by FC using a Pas III flow cytometer (Partec, Germany). Data were evaluated using WinMDI 2.8 software (Scripps Institute, La Jolla, USA).

Statistical analysis

All results were analysed by one way-ANOVA and Bonferroni's test. The analysis was performed using Prism software (Graph Pad, San Diego, CA, USA). P values < 0.05 were regarded as statistically significant.

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Abbreviations:

ECM, extracellular matrix; MDR, multidrug resistance; HA, hyaluronan; GAG, glycosaminoglycan; HAS, hyaluronan synthases; HYAL, hyaluronidase; CD44, cluster differentiation 44; RHAMM, receptor for hyaluronan-mediated motility; HARE, hyaluronan receptor for endocitosis; TLR, toll like receptor; Lyve-1, lymphatic vessel endothelial hyaluronan receptor-1; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; pAkt, phosphorylated Akt; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; oHA, hyaluronan oligomers; Pgp, P-glycoprotein; CML, chronic myeloid leukaemia; Ph, Philadelphia chromosome; SA-β-gal, senescence associated β-galactosidase; SAHF senescence associated heterochromatin foci; VCR, Vincristine; 4MU, 4-Methylumbelliferone; FBS fetal bovine serum; HMW, high molecular weight; LMW, low molecular weight; FDA, Fluorescein diacetate; PI, propidium iodide; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; bHABP, biotinilated-hyaluronan binding protein; Ab, antibody; MAb, monoclonal antibody; Av-PE, Avidin-Phycoerythine; PBS, Phosphate Buffered Saline; FC, flow cytometry; IIF, Indirect Immunofluoresence; WB, western blot; ELISA, Enzyme-linked immunosorbent assay; PFA, paraformaldehyde; DAPI, 4',6-diamidino-2-phenylindole; CsA, Cyclosporine A; SD, standard deviation.

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Legends to Figures

Figure 1. Characterization of K562 and Kv562 cell lines.

(A) HA secretion and 4MU effect on HA synthesis evaluated by ELISA-like assay. Values are expressed as means ± SD of HA concentration (ng/ml upper panel and ng/10^6cells/72h lower panel) of at least 3 independent experiments. ***p< 0.001 **p<0.01 and *p<0.05. **(B)** CD44 and RHAMM expression evaluated by IIF, magnification 400X (panel I); FC (panel II) and WB (panel III). **(C)** Analysis of HA binding by FC. Bars represent means ± SD of HA binding (%) of at least 3 independent experiments. ***p<0.001 **p<0.01 and *p<0.05 vs Untreated.

Figure 2. Effect of HA, oHA and VCR on both cell lines proliferation

(A) K562 and Kv562 cells were treated with HA, oHA or VCR for 48 and 72 h. **(B)** anti-CD44 Km81 and anti-RHAMM Abs were used to evaluate the receptors involved **(C)** Ly294002 (a PI3K inhibitor) and UO126 (a MEK inhibitor) were used to assess the signalling pathways involved. Results are expressed as:

$$\text{cell proliferation percentage} = (\text{treated cpm} \times 100 / \text{untreated cpm}) - 100$$

Bars represent means ± SD of at least 5 independent experiments ***p<0.001, **p<0.01 and *p<0.05 vs untreated.

Figure 3. Signalling pathways and receptors involved in the HA effect

(A) K562 and Kv562 cells were treated either with HA or oHA for 24 h. **(B)** Ly294002 and UO126 were used to confirm whether HA activates PI3K and/or MEK signalling pathways. **(C)** anti-CD44 Km81 was used to determine if CD44 was involved in the HA effect. **(D)** Kv562 cells were incubated with anti-RHAMM Ab to determinate whether HA induced Akt phosphorylation

mediated by RHAMM. Results are expressed as:

$$pAkt\ index = \frac{(pAkt/\beta - actin)/(Akt/\beta - actin)treated}{(pAkt/\beta - actin)/(Akt/\beta - actin)untreated}$$

or pERK/ERK index (calculated likewise). Bars represent means ± SD of at least 3 independent experiments ***p<0.001, **p<0.01, *p<0.05 and ns = no significant (p>0.05) vs untreated.

Figure 4. Effect of 4MU on K562 and Kv562 cell lines growth.

(A) Inhibition of cell growth was evaluated by [^{3}H]-thymidine uptake. Both cell lines were treated with 4MU, 4MU + HA, 4MU + HA + anti-CD44Km81 or 4MU + HA + anti-RHAMM Ab for 48 h. Results are expressed as:

$$\text{cell proliferation inhibition percentage} = 100 - (\text{treated cpm} \times 100 / \text{untreated cpm})$$

Bars represent means ± SD of at least 3 independent experiments **p<0.01 and *p<0.05 vs untreated. **(B)** K562 and Kv562 cells were treated with 4MU or 4MU + HA for 24 h. Results are expressed as:

$$pAkt\ index = \frac{(pAkt/\beta - actin)/(Akt/\beta - actin)treated}{(pAkt/\beta - actin)/(Akt/\beta - actin)untreated}$$

or pERK/ERK index (calculated likewise). Bars represent means ± SD of 3 independent experiments ***p<0.001, **p<0.01, *p<0.05 and ns = no significant (p>0.05) vs untreated.

Figure 5. Effect of 4MU on senescence induction in K562 and Kv562 cell lines

(A) To determine if 4MU induced apoptosis, both cell lines were treated with 4MU for 48 h and cellular viability was evaluated with FDA-PI by FC. Graphics show one representative experiment. **(B)** To determine if 4MU induced senescence, both cell lines were treated either with 4MU or 4MU + HA for 48 h. SA-β-gal activity (panel I); SAHF, magnification 400X,

(panel II) and cell cycle (panel III) were evaluated. Bars represent means ± SD of 3 independent experiments ***p<0.001, **p<0.01 and *p<0.05 vs untreated. Arrows point nuclei with SAHF.

Figure 6. Sensitization of Kv562 cells to the effect of VCR mediated by 4MU.

(A) Inhibition of cell growth was evaluated by [^{3}H]-thymidine uptake. Both cell lines were treated with VCR, 4MU, 4MU + VCR or 4MU + VCR + HA for 48 h. Results are expressed as:

cell proliferation inhibition percentage = 100 – (treated cpm x 100 / untreated cpm)

Bars represent means ± SD of at least 3 independent experiments ***p<0.001, **p<0.01 and *p<0.05 vs untreated. **(B)** Flow cytometry analysis of Doxorubicin efflux evaluated on K562 and Kv562 cells. Kv562 cells were treated with CsA, 4MU, 4MU + HA or 4MU + HA + anti-CD44 Km81 for 24 h. Cell lines were incubated with Doxorubicin for 40 min. Doxorubicin fluorescence was enhanced by CsA in resistant cells as a result of drug efflux blockage. Results are expressed as percentage of cells with Doxorubicin (Dox) accumulation. Bars represent means ± SD of 3 independent experiments ***p<0.001 and *p<0.05 vs untreated. **(C)** Senescence induction was assessed by SA-β-gal activity (panel I); SAHF, magnification 400X, (panel II) and cell cycle (panel III). The Kv562 cell line was treated with VCR, 4MU, 4MU + VCR or 4MU + VCR + HA. Bars represent means ± SD of 3 independent experiments ***p<0.001, **p<0.01 and *p<0.05 vs untreated. Arrows point nuclei with SAHF.

Figure 7. Sensitization of Kv562 cells to the effect of VCR mediated by oHA.

(A) Inhibition of cell growth was evaluated by [^{3}H]-thymidine uptake. Both cell lines were treated with oHA, VCR, oHA + VCR, anti-CD44 Km81, anti-CD44 Km81 + oHA, anti-CD44 Km81 + VCR, anti-CD44 Km81 + oHA + VCR, Ly294002, Ly294002 + VCR, UO126 or

UO126 + VCR for 48 h. Results are expressed as:

$$\text{cell proliferation inhibition percentage} = 100 - (\text{treated cpm} \times 100 / \text{untreated cpm})$$

Bars represent means ± SD of 5 independent experiments ***p<0.001, **p<0.01, *p<0.05 and ns = no significant (p>0.05) vs untreated. **(B)** Flow cytometry analysis of Doxorubicin efflux evaluated on K562 and Kv562 cells. Kv562 cells were treated with CsA, oHA, anti-CD44 Km81, oHA + anti-CD44 Km81 or Ly294002 for 24 h. Cell lines were incubated with Doxorubicin for 40 min. Dox fluorescence was enhanced by CsA in resistant cells as a result of drug efflux blockage. Results are expressed as percentage of cells with Doxorubicin (Dox) accumulation. Bars represent means ± SD of 3 independent experiments ***p<0.001 and ns = no significant (p>0.05) vs untreated. **(C)** Senescence induction was assessed by SA-β-gal activity (panel I), SAHF, magnification 400X (panel II) and cell cycle (panel III). The Kv562 cell line was treated with HA, oHA, VCR, oHA + VCR or HA + VCR for 48 h. Bars represent means ± SD of 3 independent experiments ***p<0.001, **p<0.01 and *p<0.05 vs untreated. Arrows point nuclei with SAHF.

Figure 8. Suggested mechanism of action of HA on K562 and Kv562 cell lines for induction of growth and MDR.

(A) Proposed mechanism of action of HA on K562 cells for induction of cell proliferation. HA-CD44 interaction might activate PI3K/Akt and MEK/ERK signalling pathways inducing cell proliferation. 4MU inhibits HA synthesis blocking signalling pathways triggered by HA, which may enhance senescence induction. oHA could block the effect of HA decreasing K562 cells proliferation. **(B) Proposed mechanism of action of HA on Kv562 cell line proliferation**. HA-RHAMM interaction would activate CD44 or other cell surface receptors triggering PI3K/Akt pathway enhancing cell proliferation. 4MU inhibits HA synthesis leading to downregulation of

PI3K/Akt pathways and induction of senescence. Both 4MU and oHA sensitized Kv562 cells to the effect of VCR through Pgp inhibition and the decrease of pAkt, enhancing senescence induction. **(C)** We suggest that high amounts of HA could favour cell proliferation, MDR and avoid senescence. Blockage of HA effect mediated by 4MU or oHA plus VCR would be able to inhibit cell proliferation and to sensitize human leukaemia cells inducing senescence.