Anti-inflammatory and anti-tumor effects of α-L-guluronic acid (G2013) on cancer-related inflammation in a murine breast cancer model

Fateme Hosseini,1, Ahmad Mahdian-Shakib,2,3,1, Farhad Jadidi-Niaragh,4,5, Seyed Ehsan Enderami,6 Hamed Mohammadizadeh,5, Maryam Hemmatzadeh,5, Hussaini Alhassan Mohammed,5, Ali Anissian,5, Parviz Kokhaei,5, Abbas Mirshafiey,2,6,7, Hadi Hassannia1

Keywords: Cancer-related inflammation, Gularonic acid, NSAIDs, Breast cancer

ABSTRACT

Cancer-related inflammation (CRI) is associated with the malignant progression of several cancer types. Targeting these pathways is a novel promising strategy for cancer prevention and treatment. In this present study, we evaluated the efficacy of α-L-guluronic acid (ALG), a potent anti-inflammatory agent on breast cancer-related inflammation both in vitro and in vivo conditions. Our results indicated that ALG can effectively inhibit the CRI and tumor-promoting mediators (COX-2, MMP2, MMP9, VEGF and proinflammatory cytokines) without direct toxic effects on the cells. Moreover, it was found that, ALG can effectively inhibit the tumor cell adhesion to extracellular matrix, seeding in implantation tissue, reduce accumulation of immunosuppressive and inflammatory cells in tumor-bearing mice. These findings were associated with decreased tumor growth, metastasis, angiogenesis and prolonged mice survival. In conclusion, our data provide a cellular and molecular justification for the use of nonsteroidal anti-inflammatory drugs (NSAIDs) in treating cancer and imply the potential anti-tumor activity of ALG therapy via inhibition of CRI. These findings could lead to the establishment of novel NSAID-based cancer therapy in the near future and open a new horizon for cancer treatment.

1. Introduction

Despite extensive research efforts, cancer still remain one of the leading causes of morbidity and mortality worldwide, with 8.2 million deaths annually [1]. The high mortality in cancer is due mainly to the metastatic spread of tumor cells into vital organs [2]. Surgery, chemotherapy, and targeted therapy are currently the main therapeutic options for breast cancer therapy, however, these approaches are not completely effective due to residual tumor cells and metastases which are often associated with serious life threatening side effects [3,4]. Several studies have shown the role of inflammation and inflammatory mediators in tumor progression, angiogenesis, metastasis, and immunosuppression [5]. The pathways that connect inflammation and cancer, causes activation of inflammatory processes, especially, NF-κB and COX-2 cascade, which are mainly overexpressed in tumor tissue [6]. COX-2 has been recognized as an upstream stimulator of inflammatory mediators such as inflammatory cytokines, adhesion molecules, prostaglandins (especially, PGE2) which enhance angiogenesis, via upregulation of VEGF, apoptosis resistance through upregulation of proto-oncogene Bcl-2, tumor growth via upregulation of EGFR, and metastasis by MMP2 and MMP9 upregulation [7]. In a malignant tissue, tumor cells recruit and instruct host immune cells to promote tumor progression. It has been shown that, more than 50% of the tumor mass is composed of macrophages that are commonly referred as tumor-associated macrophages (TAMs), neutrophils, myeloid derived suppressor cells (MDSC) and regulatory T (Treg) cells, which not only attenuate antitumor immune responses, but also they promote tumor growth, angiogenesis, lymphangiogenesis and metastasis [8–10].

1 These authors contributed equally.

Received 28 October 2017; Received in revised form 7 December 2017; Accepted 28 December 2017
0753-3322/ © 2017 Elsevier Masson SAS. All rights reserved.
evidence for the importance of cancer-related inflammation (CRI) in tumor progression comes from the effectiveness of long-term and regular use of non-steroidal anti-inflammatory drugs (NSAIDs), in preventing and treatment of colorectal, breast and other cancers [11,12]. However, the use of NSAIDs has been limited due to high side effects. Non-specific colitis, ulcer, bleeding and perforation of the colon, small intestinal inflammation, in some cases cardiovascular and renal toxicities are among these harmful effects [13,14]. Thus, it is worthwhile to investigate new anti-inflammatory drugs that are safe with more therapeutic effects for long-term use.

Small molecule G2013 (C₆H₁₀O₇; α-L guluronic acid; ALG), a novel anti-inflammatory agent with the immunomodulatory properties (G2013, patented DE-102016113017.6), is an attractive drug for cancer therapy. ALG is an epimeric form of M2000 (β-D mannuronic acid; BDM), its anti-inflammatory and anti-cancer properties have been published, previously [14]. ALG and BDM are derived from alginic acid sodium salt and exert, their anti-inflammatory and anti-metastatic potential of ALG, cell adhesion assay was performed. In brief, the 4T1 cells were pre-treated with different concentrations of ALG (0, 25, 250, 500 μg/ml) for 48 h. The supernatants were then centrifuged at 400 g for 5 min at 4 °C, and equal amounts of protein (4 μg) were electrophoresed under non-reducing conditions as previously described [14]. After electrophoresis, the gels were washed with 2.5% Triton X-100 solution to remove SDS. Then, gels were incubated overnight at 37 °C in substrate buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM CaCl₂ with 0.02% NaN₃, and stained with coomassie blue. The gel was scanned and densitometry analyses for bands were calculated using Image J 1.46r software (National Institutes of Health, Bethesda, MA, USA).

2. Materials and methods

2.1. Cell lines and mice

The cell lines were purchased from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). ALG was extracted from alginic acid sodium salt (Sigma-Aldrich) by acid hydrolysis method as described previously [15] and then dissolved in PBS (10 mg/ml) as a stock solution. The present study was designed to test anti-inflammatory and anti-tumor effects of ALG both in vitro and in vivo (a schematic demonstration of study is shown in Fig. 1). Female inbred BALB/c mice (6–8 weeks old and 18–20 g weight) were obtained from Pasteur Institute of Iran.

2.2. In vitro assessment

2.2.1. Proliferation assay

To investigate the effects of ALG on cell proliferation, 4T1 cells were plated overnight in 96-well plates at a density of 5 × 10³ cells/well. The medium was replaced with the same media containing different concentrations of ALG (0, 25, 50, 250, 500 μg/ml). Plates were incubated at 37 °C for 48 h and then the cells were pulsed with 1 μCi of [3H]-thymidine (PerkinElmer, Boston, USA) for 18 h. The cells were harvested and transferred to scintillation fluid for measurement of radioactive thymidine incorporation by a beta counter (Wallac 1410 Liquid Scintillation Counter, Pharmacia, Sweden).

2.2.2. Gelatin zymography

This electrophoretic technique was used to test gelatinolytic activities of MMP2 and MMP9 which are associated with metastasis potential of tumor cells. The 4T1 cells were treated with different concentrations of ALG (0, 25, 250, 500 μg/ml) in serum-free medium for 48 h. The supernatants were then centrifuged at 400 g for 5 min at 4 °C, and equal amounts of protein (4 μg) were electrophoresed under non-reducing conditions as previously described [14]. After electrophoresis, the gels were washed with 2.5% Triton X-100 solution to remove SDS. Then, gels were incubated overnight at 37 °C in substrate buffer containing 50 mM Tris-HCl, pH 8.0, 5 mM CaCl₂ with 0.02% NaN₃, and stained with coomassie blue. The gel was scanned and densitometry analyses for bands were calculated using Image J 1.46r software (National Institutes of Health, Bethesda, MA, USA).

2.2.3. Adhesion assay

In order to evaluate the in vitro anti-metastatic potential of ALG, cell adhesion assay was performed. In briefly, the 4T1 cells were pre-treated with different concentrations of ALG (0, 25, 50, 250, 500 μg/ml) for 48 h, and then trypsinized. 5 × 10³ cells/well were seeded in triplicate into 96-well plates which were precoated with collagen I, fibronectin and laminin (Sigma-Aldrich). Plates were incubated for 2 h at 37 °C. The non-adherent cells were removed by gentle washing and adherent cells were fixed with 100% ethanol and stained with 0.1% crystal violet (Sigma-Aldrich). At the next step, the precipitates were dissolved by the

---

**Fig. 1.** Experimental design scheme. (A) The chemical structure of ALG (molecular weight, 194.139 g/mol) (B) The schematic design of the in vitro study. (C) Schematic design of the in vivo study, animals were divided into 3 groups (ten mice per group): treated with PBS (group 1); treated with 50 μg/kg of ALG after tumor induction (group 2); or inoculated with 4T1 cells treated with 50 μg/ml of ALG (group 3). Tumor growth was measured every 2 days.
addition of 10% acetic acid, and the absorption was observed at 570 nm.

2.2.4. Evaluation of COX-2 activity
To assess the anti-inflammatory potential of ALG, we evaluated the secretion of COX-2-derived PGE2 in supernatants of LPS-activated murine macrophage J774 cell line. J774 cells were plated 4 h in 24-well plates at a density of 2.5 × 10^5 cells/well. The media were then collected and centrifuged 24 h after treatment with 1 μg/ml of LPS in the presence of different concentrations of ALG (0, 25, 250, 500 μg/ml). PGE2 was measured by commercially available ELISA kit (PE2, R&D Systems, Minneapolis, Minnesota, USA), according to the manufacturer protocol.

2.3. In vivo assessment

2.3.1. Experimental cancer model establishment and treatment
The effects of ALG on CRI in a physiological environment, was performed using the 4T1 syngeneic breast cancer model. 30 BALB/c mice were randomly assigned to three independent groups (ten mice per group):

(1) Control group (treated with PBS), (2) Therapeutic group (treated with intraperitoneally 50 mg/kg of ALG after tumor induction), (3) Cell line pre-treated group (4T1 cells treated with 50 μg/ml of ALG before tumor inoculation) (Fig. 1C). The effective doses of ALG was assigned to groups based on our previous findings [14]. After acclimatization, tumors were induced by injecting 4T1 cells (5 × 10^5) into the mammary fat pad. The PBS and ALG treatment was initiated after six days when the tumors sizes reached 30–50 mm³ in group 1 and 2, respectively. The tumor sizes were measured every 2 days using digital callipers and body weight was recorded weekly. After 30 days of inoculation, mice (n = 5 in each group) were sacrificed and the tumors, selected internal organs such as lungs, liver, spleen, heart, kidneys, brain, tumor regional lymph nodes and blood samples were collected for histology, flow cytometry, gene expression, haematological and serum cytokine assay. The overall survival analysis was also performed using five mice in each group.

2.3.2. Histological analysis
In order to investigate the tumor histomorphometric characterization and incidences of metastases to various organs, all dissected organs were fixed in 10% buffered formalin and embedded in paraffin using standard methods. Three μm serial sections were taken from each tissue and stained with hematoxilin and eosin (H&E). Then, sections were scanned using a digital slide scanner (Panoramic DESK, 3D Histech, Hungary). Moreover, the percentage of lung metastatic area per section was calculated with the Panoramic Viewer software.

2.3.3. Tissue preparation and analysis of MDSC and Treg cells
Spleens, tumors and lymph nodes were gently dissociated under 40 μm mesh cell strainer (BD Biosciences) for single-cell isolation [21]. After red blood cell lysis, the cells were washed and labelled with fluorescence-conjugated antibodies against mouse Gr-1-APC and CD11b-PE (BD, Pharmingen) to identify MDSC, or with CD4-APC and FoxP3-Alexa Fluor 488 to identify Treg cells and isotype-matched controls. For intracellular staining, cells were fixed and permeabilized with Foxp3 Fix/perm buffer before incubation with the conjugated antibody or isotype control. Samples were analysed with a FACSCalibur flow cytometer (Becton Dickinson) and data was analyzed using Flowjo software (version 7).

2.3.4. Hematological and cytokines analysis
Total white blood cells (WBC) count was performed using Neubauer chamber. The smears were stained with Giemsa solution and viewed for the presence of the granulocyte, lymphocyte and monocyte populations. The serum levels of IL-1β, IL-6, and TNF-α cytokines were measured by commercially available ELISA kits (R&D, USA), according to the manufacturer’s instructions.

2.3.5. Gene expression analysis by real-time PCR
To better define the underlying mechanisms of the anti-tumor effects of ALG, we compared mRNA expression of major factors that promote inflammation (COX-2), angiogenesis (VEGF) and metastasis (MMP2 and MMP-9) in tumor tissues from each group. Total RNA was extracted using GeneAll RiboEx kit (GeneAll, Korea) and subsequently converted into complementary DNA by high-capacity cDNA reverse transcription kit (Applied Biosystems, USA) and the PCR assay was performed by using the SYBR Green fluorescence quantification system (Takara). The primer sequences used were as follows: COX-2, F: TCTTGACAAGGGTGGGAACCT and R: TAGATGGCCACTGCACTTGT; MMP2, F: GGCGACTGAAATCTGAACACT and R: GTCTGGGCCAGCCAAA GAAC; MMP9, F: ACACGACATCTCCAGTACC and R: CAGGAGCT CGTAAAGTCAGTACG; VEGF, F: GAGCAGAACCTCCCAGTAAGTGA and R: CACCGAGCGCTGGTAAGATGT; β-actin, F: GGTCATCATTAGGCA AGC and R: ACAGTTGCAAGTGCACACT. The specificity of gene-specific amplification was confirmed by melting curve analysis. Relative mRNA levels were quantified using the 2^-ΔΔCT method. All reactions were performed in triplicate using β-actin as an internal control.

2.3.6. Statistical analysis
Statistical analysis was performed using the SPSS statistical package (SPSS, Chicago, IL, USA) and GraphPad Prism (San Diego, California, USA). Two group comparisons were assessed with a Mann-Whitney U test. Comparisons between groups were investigated by one-way ANOVA analysis. Kaplan-Meier survival curves were analyzed using log-rank tests. The correlation analysis was performed using Pearson and Spearman correlation tests. Where appropriate. Data are presented as a mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 were considered statistically significant difference.

3. Results

3.1. ALG therapy effectively inhibits activity of MMPs, COX-2 and adhesion of tumor cell lines in in vitro assessment
To determine direct and indirect anti-tumor effects of ALG, tumor cells were incubated with various concentrations (0, 25, 250, 500 μg/ml) of ALG for 48 h. Proliferation assay showed that even at the high concentration of 500 μg/ml of ALG there were no significant changes in the tumor cell proliferation, in comparison to the control. (Fig. 2A).

Furthermore, the results of the trypan blue exclusion test have indicated similar results (Fig. 2B), there was low direct cytotoxicity of ALG on tumor cells in vitro. The anti-tumoral and anti-metastatic potential of ALG, was performed by gelatinolytic capacity of tumor cells (zymography) and the ability of tumor cells to bind extracellular matrix proteins (adhesion assay). Our results revealed a significant decrease in the activity of MMP2 and MMP9 compared to untreated cells (Fig. 2C).

Based on the relative band intensity, ~22–31% activity of MMP2 (Fig. 2D) and ~28–44% activity of MMP 9 (Fig. 2E) in 4T1 cells were decreased in response to treatment with ALG in a dose-dependent manner. Furthermore, ALG treatment significantly reduces the adhesion capacity of tumor cells to collagen I, fibronectin, and laminin which are the major components of the extracellular matrix (ECM) (Fig. 2F).

The anti-inflammatory potential of ALG, was assessed by evaluating the secretion of COX-2 derived PGE2 in the supernatants of J774 cell line in response to LPS in the absence or presence of ALG (Fig. 2G). The result showed that the level of PGE2 in ALG treated group ~66% was lesser than LPS group in all the doses used.

Biomedicine & Pharmacotherapy 98 (2018) 793–800

795
3.2. ALG therapy inhibits tumor growth, metastasis and prolonged tumor-bearing mice survival time

Following assessment of the in vitro anti-inflammatory and anti-tumoral effects of ALG, it was necessary to determine whether ALG is capable of reducing CRI and inhibits tumor progression during tumor challenge. Based on low concentrations, no toxicity, significantly reduce the activation of COX-2, MMPs and adhesion, which is the recommended for clinical trial, we assigned dose of 50 mg/kg (Therapeutic group) and 50 μg/ml (Cell line pre-treated group) as a suitable ALG dose for the in vivo study. Six days after implantation, the primary tumor appeared in all the mice. As demonstrated in Fig. 3A, tumor volumes in therapeutic group ∼ 61.4% and cell line-treated group ∼ 94.1% were smaller than the control group on the 30th day after tumor inoculation. Also, ALG therapy was found to significantly reduced tumor growth rate (Fig. 3B). The maximum reduction in the tumor volume and arrested tumor growth rate was observed in cell line treated model. The follow up on all the mice up to 90 days after tumor inoculation showed a similar pattern to what was observed in the first 30 days after tumor injection (Fig. 3C). It was found that ALG therapy did not cause a significant change in body weight, this is an indication that there was no change in animal appetite on water, food consumption and no general toxicity in ALG treated groups (Fig. 3D).

To evaluate whether ALG therapy inhibits the tumor metastasis in the murine breast cancer model, H&E stained sections were analyzed (Fig. 3E). All the mice in control group had metastases, the incidence of metastasis in the lungs (100%), spleen (60%), tumor regional lymph nodes (80%), liver (80%), Brain (20%), and with total absence of metastases in the other dissected organs (Fig. 3F). In the therapeutic group, metastasis in the lung (80%), tumor regional lymph nodes (60%), liver (20%) and no observed metastases in the other dissected organs. Surprisingly, there was no metastasis in all the organs in cell line treated group. This may be due to decreased tumorigenicity of cancer cells (MMP activity and adhesion to ECM components) treated with 50 μg/ml of ALG before tumor inoculation, which partially inhibits tumor cell seeding in the implantation site. We also observed a significant positive correlation between the tumor size and the number of organ metastasis (Fig. 3G). As expected, reduced cancer cell dissemination was associated with a decrease in the area of lung metastasis in ALG treated groups compared to the control group (Fig. 3H). Lung metastasis area in control group (∼ 15% per section), therapeutic group (∼ 5% per section) and there was no metastasis in the cell line treated group. Moreover, ALG significantly increased the survival time of tumor-bearing mice compared to control group (Fig. 3I).

3.3. ALG therapy decreased inflammation and immunosuppressive cells in tumor-bearing mice

The concentrations of IL-1β, IL-6, and TNF-α in serum, WBC count and their subtypes in tumor-bearing mice was evaluated in order to investigate the effects of ALG on systemic inflammation. The results showed that the concentrations of IL-1β (Fig. 4A) and IL-6 (Fig. 4B) were significantly lower in ALG-treated groups, compared to control group, whereas TNF-α levels showed no difference between all three groups (Fig. 4C). Moreover, the increase of pro-inflammatory cytokine was accompanied by increased WBC count (Fig. 4D) and higher
Neutrophil to lymphocyte ratio was observed (which is an indicator of poor prognosis in cancer) (Fig. 4E). These results were clearly confirmed by the high frequency of inflammatory cells (particularly, granulocytes) within tumor blood vessels in control group compared to ALG treated groups by tumor histological analysis (Fig. 4F).

To evaluate the effects of ALG on the local accumulation of immunosuppressive cells in tumor-bearing mice, the Treg and MDSC frequencies in the tumors, spleens, and tumor regional lymph nodes were evaluated in each group. Representative dot plots was used for the enumeration of Treg as shown in (Fig. 4G) and MDSCs (Fig. 4H). Interestingly, ALG therapy was found to significantly reduce the frequency of Treg cells in tumor tissues and lymph node as compared to control group (Fig. 4I), and ALG therapy has significantly decreased MDSCs in tumor and in spleen (Fig. 4J). Although the frequency of Treg in spleen and frequency of MDSC in lymph node was reduced in ALG treated groups, these reductions were not statistically significant. However, the frequencies of Treg and MDSC in tumor-bearing mice were significantly correlated with tumor size (Fig. 4K). Due to the small size of the primary tumor in ALG cell line pre-treated group, we were unable to determine the frequency of these cells in tumor tissue.

3.4. ALG therapy suppressed angiogenesis in the tumor microenvironment through inhibition of CRI

Histological analysis of tumor tissue has shown significant differences in the tumor microenvironment in the ALG treated groups compared with control group, on the 30th day after tumor inoculation (Fig. 5A). The number of blood vessels (angiogenesis) in therapeutic
group ~ 59.8% and cell line-treated group ~ 91.1% were lesser than the control group (Fig. 5B). In the control group, high levels of mitosis (averaging 4–6 mitosis/high-power fields [hpf]), high necrosis area in the center of the tumor, which was presumably due to hypoxia and high leukocyte infiltration, which was mainly composed of TAMs with foamy cytoplasm, some granulocytes and lymphocytes were observed. While in the therapeutic group, the low rate mitosis (3 mitosis/hpf), small necrosis area and low level of infiltration leukocyte were observed.

Surprisingly, in cell line-treated group the mitotic rate was very low (> 1 mitosis/5 hpf) and no visible infiltrated inflammatory cells and necrosis area were observed, this may be in part due to the failure of tumor cell seeding. As expected, analysis of RT-qPCR data has indicated that, the expressions of inflammatory and tumor-promoting factors (COX-2, MMP2,9 and VEGF) in ALG treated groups were significantly lower than that in the control group (Fig. 5C).
activity of in... of PGE2 as the main product of COX-2 according to ECM components) were strongly altered after ALG treatment. ALG did not have a direct effect on tumor cell proliferation, although the decrease in tumor size in ALG treated groups was correlated with reduced accumulation of inflammatory cells in tumor-bearing mice (Fig. 4). Our results showed that ALG therapy could significantly reduce the frequency of MDSC (in tumor tissues and spleen) and Treg cells (in tumor tissues and lymph node) compared to control group. On the other hand, the increase of neutrophil to lymphocyte ratio has been used as a marker for systemic inflammatory status, this concept is supported by the high concentration of IL-1 and IL-6 in the serum of control mice, compared with ALG treated groups. In parallel, histological analysis revealed high levels of mitosis, leukocyte infiltration and angiogenesis in control mice compared to ALG treated groups (Fig. 5A, B). This difference may be due in part to low expression of inflammatory and tumor-promoting factors (COX-2, MMP2,9 and VEGF) in ALG treated groups (Fig. 5C). Although the ALG molecular mechanism is not clearly elucidated, but was thought that, similar to other NSAIDs the main mechanism of action of ALG consists of inhibiting prostaglandin synthesis by blocking the COX-2 cascade (Fig. 5D), however, experimental data strongly indicates the existence of additional mechanisms. Our study shows that ALG can partially inhibit the tumor cell adhesion to extracellular matrix and disrupt the tumor cell seeding in implantation tissue in cell line treated group. According to Steven Paget “Seed and Soil” hypothesis, the minimum tumor incidence, volume, and metastasis in group 3 may be associated with interference in the interaction between tumor cells and surrounding microenvironment.

In conclusion, our results provide a cellular and molecular justification for the use of NSAIDs in treating cancer and shows the anti-tumor activity of ALG therapy via inhibition of CRI. This therapeutic pattern can be repeated, however, all of these suggestions should be precisely examined in the initial trials. It should be noted that the addition of other therapeutic approaches such as surgery, chemotherapy, and targeted therapy to a combination of ALG therapy may lead to a better therapeutic outcome which will be the goal of future studies.

Fig. 5. Effect of ALG on CRI in the tumor microenvironment. (A) Representative fields of angiogenesis (measured as blood vessels per mm²). (B) The relative mRNA expression of major factors that promote angiogenesis and metastasis in cell treated group. (C) The relative mRNA expression of major factors that promote angiogenesis and metastasis in the tumor microenvironment. (D) Schematic role of CRI in the promotion of tumorigenesis.

4. Discussion

The association between cancer and inflammation development was identified in 1863 by Rudolf Virchow [22]. Over the past decades, epidemiological studies have shown that CRI plays a critical role in tumor progression, angiogenesis, metastasis, and immunosuppression [23]. Hence, there has been growing interest in the use of NSAIDs for cancer prevention and treatment [24]. However, all the available NSAIDs are associated with serious toxicity effects and several reports have described the anti-tumor activity of NSAIDs is lower than the common anticancer drugs [25]. Results of our study and other researchers, have considered ALG as a novel NSAID with high immunomodulatory properties and anti-inflammatory effects, coupled with high tolerability and safety. Recent observations have indicated that ALG can regulate TLR2 and TLR4 signalling pathways [26,27], reducing the production of nitric oxide, PGE2, reactive oxygen species, expression of COX-2, secretion of proinflammatory cytokines [17,28–30], and regulation of expression of enzymes involved in oxidative stress including superoxide dismutase, catalase, glutathione peroxidase, glutathione s-transferase and myeloperoxidase [31,32]. Moreover, several studies have shown that the main biological obstacles in solid tumor therapy are poor penetration and acidic microenvironment, which inhibit drugs uptake and function on tumor cells [33,34]. It seems that the low molecular size and ionic acid structure of ALG enable it to overcome these obstacles (Fig. 1A). In the present study, we investigated the effects of ALG on CRI in both in vitro and in vivo. The in vitro results showed that ALG can effectively inhibit the activity of inflammatory and tumor-promoting mediators in a dose dependent manner without toxic effects. This observation suggests that ALG did not have a direct effect on tumor cell proliferation, although the tumorigenicity properties of these cells (MMP activity and adhesion to ECM components) were strongly altered after ALG treatment (Fig. 2A–G). The effects of ALG on activation of the COX-2 in LPS-activated murine macrophage J774 cell line was also examined. We determined the concentration of PGE2 as the main product of COX-2 activation in each sample. The result showed that the level of PGE2 in ALG treated group was significantly lower than LPS group (Fig. 2E). Interestingly, the in vivo results showed that ALG therapy strongly inhibits CRI in tumor bearing mice. Inhibition of CRI was associated with decreased tumor growth, metastasis, angiogenesis and prolonged survival (Fig. 3). In contrast, the high metastasis rate in group 2 (80% to lung and 60% to tumor regional lymph nodes) might be due to 16 days gap between tumor inoculation and visible reduction in tumor size. We started ALG therapy six days after tumor injection and ten days after that (~16th days) visible reduction in tumor size was observed. This period provides the required time for 4T1 tumor cells spreads through the blood or lymphatic vessels [35]. Consistent with the previous findings, we found that the decrease in tumor size in ALG treated groups was correlated with reduced accumulation of immunosuppressive and inflammatory cells in tumor-bearing mice (Fig. 4). Our results showed that ALG therapy could significantly reduce the frequency of MDSC (in tumor tissues and spleen) and Treg cells (in tumor tissues and lymph node) compared to control group. On the other hand, the increase of neutrophil to lymphocyte ratio has been used as a marker for systemic inflammatory status, this concept is supported by the high concentration of IL-1 and IL-6 in the serum of control mice, compared with ALG treated groups. In parallel, histological analysis revealed high levels of mitosis, leukocyte infiltration and angiogenesis in control mice compared to ALG treated groups (Fig. 5A, B). This difference may be due in part to low expression of inflammatory and tumor-promoting factors (COX-2, MMP2,9 and VEGF) in ALG treated groups (Fig. 5C). Although the ALG molecular mechanism is not clearly elucidated, but was thought that, similar to other NSAIDs the main mechanism of action of ALG consists of inhibiting prostaglandin synthesis by blocking the COX-2 cascade (Fig. 5D), however, experimental data strongly indicates the existence of additional mechanisms. Our study shows that ALG can partially inhibit the tumor cell adhesion to extracellular matrix and disrupt the tumor cell seeding in implantation tissue in cell line treated group. According to Steven Paget “Seed and Soil” hypothesis, the minimum tumor incidence, volume, and metastasis in group 3 may be associated with interference in the interaction between tumor cells and surrounding microenvironment.
Also, regarding the high safety of ALG, it is worthwhile to use it with the preventive approach in higher-risk people with a family history of cancer.

Compliance with ethical standards

All experiments involving mice were approved by the ethics committee of Tehran University of Medical Sciences.

Conflict of interest

The authors have no financial conflict of interest.

Acknowledgements

This work was supported by a grant from Tehran University of Medical Sciences (No. 33352) and Semnan University of Medical Sciences (No. 1003).

References