Alternagin-C binding to α₂β₁ integrin controls matrix metalloprotease-9 and matrix metalloprotease-2 in breast tumor cells and endothelial cells

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Abstract

Background: Matrix metalloproteinases (MMPs) are key players in tumor progression, helping tumor cells to modify their microenvironment, which allows cell migration to secondary sites. The role of integrins, adhesion receptors that connect cells to the extracellular matrix, in MMP expression and activity has been previously suggested. However, the mechanisms by which integrins control MMP expression are not completely understood. Particularly, the role of α₂β₁ integrin, one of the major collagen I receptors, in MMP activity and expression has not been studied. Alternagin-C (ALT-C), a glutamate-cysteine-aspartate-disintegrin from Bothrops alternatus venom, has high affinity for an α₂β₁ integrin. Herein, we used ALT-C as a α₂β₁ integrin ligand to study the effect of ALT-C on MMP-9 and MMP-2 expression as well as on tumor cells, fibroblasts and endothelial cell migration.

Methods: ALT-C was purified by two steps of gel filtration followed by anion exchange chromatography. The α₂β₁ integrin binding properties of ALT-C, its dissociation constant (K_d) relative to this integrin and to collagen I (Col I) were determined by surface plasmon resonance. The effects of ALT-C (10, 40, 100 and 1000 nM) in migration assays were studied using three human cell lines: human fibroblasts, breast tumor cell line MDA-MB-231, and microvascular endothelial cells HMEC-1, considering cells found in the tumor microenvironment. ALT-C effects on MMP-9 and MMP-2 expression and activity were analyzed by quantitative PCR and gelatin zymography, respectively. Focal adhesion kinase activation was determined by western blotting.

Results: Our data demonstrate that ALT-C, after binding to α₂β₁ integrin, acts by two distinct mechanisms against tumor progression, depending on the cell type: in tumor cells, ALT-C decreases MMP-9 and MMP-2 contents and activity, but increases focal adhesion kinase phosphorylation and transmigration; and in endothelial cells, ALT-C inhibits MMP-2, which is necessary for tumor angiogenesis. ALT-C also upregulates c-Myc mRNA level, which is related to tumor suppression.

Conclusion: These results demonstrate that α₂β₁ integrin controls MMP expression and reveal this integrin as a target for the development of antiangiogenic and antimetastatic therapies.

Keywords: ALT-C, α₂β₁ integrin, Cancer, Tumor microenvironment, MMP, C-Myc

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Background
Metastasis is the main cause of death of patients with breast cancer; therefore, a full comprehension of the cell motility process is crucial for understanding how tumor dissemination occurs throughout the body [1]. The metastatic process involves several steps such as extracellular matrix (ECM) degradation, invasion, transendothelial cell migration, survival in circulation, extravasation, and colonization in a new site [2, 3]. The tumor stroma plays a fundamental role in tumorigenesis. It consists mainly of fibroblasts, ECM, vasculature, inflammatory cells, and mesenchymal stem cells that, in concert with tumor cells, are responsible for secreting growth factors, proteases and chemokines to induce a continuous remodeling of the tumor microenvironment [4–6].

The ECM components play key roles in intracellular signaling by interacting with adhesion receptors such as integrins [7]. Integrins comprise a family of cell adhesion heterodimeric transmembrane receptors and their expression may vary greatly according to the environment [8–11]. αβ1 integrin is one of the major receptors for type I collagen (Col I) and it has been demonstrated to be relevant during the process of bone metastasis of prostate and breast cancer [12, 13]. The role of αβ1 integrin in the tumor microenvironment has not been fully elucidated, but the expression of β1 integrin subunit is altered in 30–50% of breast tumors. The β1 integrin subunit was shown to be required for cell proliferation, survival and invasiveness of transformed cells [14–16]. However, in vitro experiments performed on primary epithelial tumor cells have shown that the deletion of αβ1 integrin increased tumor cell invasion and anchorage-independent growth [17].

Matrix metalloproteinases (MMPs) modify the microenvironment during tumor progression by inducing extracellular matrix remodeling and the release of cytokines and growth factors [18]. Overexpression of MMP-2 and MMP-9 is observed in various types of cancer, such as colorectal tumors, melanoma and breast cancer [19–21]. Moreover, MMP-2 and MMP-9 have been associated with tumor progression and decreased survival [22]. It has been recently demonstrated that active MMP-2 regulates vascular endothelial growth factor A (VEGF-A) expression in melanoma cells on a transcriptional level via an αβ1 integrin/phosphoinositide-3-kinase–(PI3K) dependent pathway [20], which results in activation of the endothelium, an essential step for the adhesion of circulating tumor cells. Therefore, integrin inhibition shows up as an interesting alternative for metastasis prevention.

Disintegrins are integrin inhibitors found in snake venoms [23]. Most disintegrins have the arginine-glycine-aspartate (RGD) motif, which is a well-known ligand of αβ integrins. However, snake venoms also have another class of integrin binding proteins in which a glutamate-cysteine-aspartate (ECD) sequence replaces the RGD motif. This special class of proteins binds to αβ integrins and competitively inhibits cell binding to Col I [24]. Alternagin-C (ALT-C), an ECD-disintegrin-like protein, has been shown to be a potent inhibitor of collagen-induced adhesion through αβ integrin inhibition [24–26]. However, the effects of ALT-C on MMPs in tumor and normal cells have not been determined. Here, we provide further evidence that ALT-C binding to αβ integrin decreases MMP-9 and MMP-2 content in human breast cancer cells and decreases MMP-2 content in human microvascular endothelial cells (HMEC-1) by zymography. The decrease in MMP-9 mRNA level was also confirmed by polymerase chain reaction (PCR) analysis. ALT-C also induces focal adhesion kinase (FAK) phosphorylation and upregulates c-Myc mRNA levels in MDA-MB-231 tumor cells. Fibroblasts were insensitive to ALT-C. These results provide new information on the roles of αβ integrin binding in the tumor cell and in its microenvironment.

Methods
Purification of alternagin-C
ALT-C was purified from Bothrops alternatus venom (donated by the Butantan Institute, São Paulo, Brazil) by two steps of gel filtration followed by anion exchange chromatography as previously described [24]. The purity of the final preparation was confirmed by mass spectrometry and it showed no residual proteolytic activity.

Surface plasmon resonance (SPR)
To better characterize the αβ integrin binding properties of ALT-C, its dissociation constants (Kd) relative to this integrin and to collagen I (Col I) was determined by surface plasmon resonance (SPR). The αβ integrin (R&D Systems) diluted in acetate buffer (20 μg/mL), pH 4.0, was immobilized to the dextran matrix of a CM5 sensor chip™ (GE Healthcare Life Sciences, Sweden) at a flow rate of 15 μL/min. This procedure resulted in ~1600 resonance units (RU). Collagen type 1 (BD Biosciences, USA) diluted in acetate buffer (30 μg/mL), pH 4.5, was similarly immobilized to the dextran matrix of a CM5 sensor chip™ at a flow rate of 15 μL/min and this procedure resulted in ~4000 RU.

Surfaces were activated and blocked using N-ethyl-N-(dimethylaminopropyl) carbodiimide plus N-hydroxysuccinimid and ethanolamine chemistry. The chip was regenerated with Gly-HCl 2 M, pH 2.0, for 10 s. ALT-C was immediately diluted in phosphate-buffered saline (PBS – 0.05–10 μM) and injected consecutively at flow rates of 15 μL/min at 25 °C using PBS as flow buffer. Measurements were performed using equipment and supplies from BIACore T200 (GE Healthcare Life Sciences, Sweden) and the BLA evaluation software. Kinetic parameters were analyzed using the 1:1 binding model by GraFit 7 software (Erithacus Software, England).
Cell lines and culture
Human fibroblasts were purchased from the Cell Bank of Rio de Janeiro (Brazil) and the human breast tumor cell line MDA-MB-231 from American Type Culture Collection (ATCC, Manassas, USA). Both cell lines were maintained in Dulbecco’s Modified Eagle Medium (DMEM – Vitrocell, Brazil) supplemented with 10% (v/v) fetal bovine serum (FBS). Human dermal (foreskins) microvascular endothelial cells HMEC-1 from (ATCC CRL-3243) were cultured in MCDB-131 (Sigma, Brazil). All cell lines were cultured in the presence of penicillin (100 IU/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM) in a humidified environment with 5% CO₂ at 37 °C. For the cell passages, 0.25% trypsin (Sigma-Aldrich, USA), 0.1% ethylenediamine tetraacetic acid (EDTA – Sigma-Aldrich, USA) solution was used.

Flow cytometry analysis
The profile of α2 and β1 integrin subunits of each cell line was determined by flow cytometry using specific monoclonal antibodies: anti-α2 (LSBio-C188740, USA), and anti-β1 (SC-13590, Santa Cruz Biotechnology). Briefly, 1 × 10⁶ cells were incubated with 1 μg of antibodies at 4 °C for 30 min. Then, cells were washed with PBS and centrifuged at 4 °C for 10 min at 150×g. Next, 0.5 μg of fluorescein isothiocyanate anti-IgG (SC-2010, Santa Cruz Biotechnology) was added to each sample and incubated for 30 min at 4 °C in absence of light. After, cells were washed again with PBS, centrifuged and immediately analyzed in a FACSCalibur flow cytometer (BD Bioscience, USA).

Transendothelial migration assay
This assay mimics tumor cell migration through endothelial blood cells, one of the crucial steps in metastasis. HMEC-1 cells were seeded (1 × 10⁵) in 8-μm pore inserts (12 wells/plate) (BD Biosciences, USA) and cultured in medium containing 10% FBS until they achieved confluence (48 h) and formed monolayers. During this period, the wells under inserts contained medium without FBS. MDA-MB-231 cells were stained with PKH26 red fluorescent cell linker (Sigma-Aldrich, USA), treated or not with ALT-C and placed in the upper chamber covered with a monolayer of HMEC-1.

The stained tumor cells were placed in the inserts with medium without FBS and under the inserts was added medium containing 5% FBS (chemoattractant, Vitrocell, Brazil). After incubation for 16 h at 37 °C, 5% CO₂, the transmigrated cells were fixed with 3.7% formaldehyde and stained with DAPI. Ten randomly chosen fields in the inserts were photographed and cells counted using a fluorescence microscope (Olympus U-RFL-T, 20× objective, DP2BSW software, Japan).

Wound healing migration assay
A wound healing migration assay measures the repopulation of wounded cultures. Cells were seeded into 12-well culture plates at 1 × 10⁵ cells/well and cultured in medium containing 10% FBS to achieve monolayer confluence. The monolayers were carefully wounded using a 200-μL pipette tip, and cellular debris was removed by washing with medium. The wounded monolayers were then incubated for 24 h in serum-free medium (SFM) containing 0–1000 nM of ALT-C. Images immediately after wounds (t = 0 h) were captured to record the initial area, and the recovery of the wounded monolayers due to cell migration toward the denuded area was evaluated at 24 h (t = Δ h).

The images were captured using an inverted microscope (Olympus CK2 ULWCD 0.30; 10× objective, Japan) equipped with a digital camera (Cool SNAP-Pro Color with Image Pro software). The area of the wound was quantified using Java’s Image J software (http://rsb.info.nih.gov) in the polygon selection mode. The migration of cells toward the wound was expressed as a percentage of wound closure: percent of wound closure = [(At = 0 h – At = Δ h)/At = 0 h] × 100%, where At = 0 h is the area of the wound measured immediately after scratching, and At = Δ h is the area of the wound measured 24 h after scratching.

Gelatin zymography assay
The content of MMPs on conditioned media from the wound healing assay was analyzed by gelatin zymography as previously described [27] with some modifications. After treatment with ALT-C, the culture medium was collected, centrifuged at 10,000×g for 5 min at 4 °C and incubated in sample buffer under non-reducing conditions. The samples were maintained in ice and immediately loaded (20 μg) in the gels. The samples were resolved on a 10% polyacrylamide gel containing 0.1% gelatin at 4 °C. The gel was washed two times with 2.5% Triton X-100 and incubated at 37 °C for 18 h in 50 mM Tris buffer, pH 8.0, 5 mM CaCl₂, 0.02% NaN₃ and 10 mM ZnCl₂. After staining with Coomassie Blue R-250 and destaining with acetic acid:methanol:water (1:4:5), the clear bands were quantified by densitometry using Image J software. MMP-2 and MMP-9 were quantified in arbitrary units (AU) using GraphPad Prism 5.0 software (La Jolla, USA).

Isolation of total RNA and synthesis of cDNA
Cells were seeded in 6-cm dishes (Corning, USA) in culture medium (DMEM or MCDB-131, Brazil) plus 10% FBS for 48 h at 37 °C and 5% CO₂. The cells were then incubated with 10, 100 or 1000 nM ALT-C. After 24 h, culture medium was removed and cells were lysed with cold TRIzol Reagent (Invitrogen, USA) according to the
manipulator’s protocol for total RNA isolation. RNA concentrations and purity were determined by the ratio of the absorbance at 260 and 280 nm using a Nanodrop 2000 the RNA integrity was confirmed on 1% agarose-formaldehyde gel stained with ethidium bromide.

Total RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase (Promega, USA). cDNA was stored at −20 °C until use. Oligonucleotide primers were designed using Primer Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The primer sequences were: GAPDH forward 5′-GATGCTGGTGTGCTTAGTATGT and reverse 5′-GTGGTCAGGATGCATTGCT; c-Myc forward 5′-CCTACCTCTCAAACGACGC and reverse 5′-CTTGTTCTCCTCAAGATCGC; MMP-2 forward 5′-AGGACCGTTCTATTGCGG and reverse 5′-TGGCT TGGGATACCTCTGC; MMP-9 forward 5′-GCTAC CACCTGAACCTT and reverse 5′-GCCATTCAAGT CGTCCATTAT.

Analysis by quantitative polymerase chain reaction (qPCR)
The amplification mixtures contained 6.25 μL of the fluorescent dye Eva Green Supermix (Bio-RadUSA), 0.5 μL of cDNA, 4.75 μL of diethyl dicarbonate (DEPC) water and 1 μL (100 μM/μL) of each primer in a final volume of 12.5 μL. Thermal cycling conditions for all genes were 15 min at 95 °C followed by 45 cycles of 30 s at 72 °C and 30 s at 56 °C for GAPDH, 59.5 °C for c-Myc, 60 °C for MMP-2 and 59 °C for MMP-9, respectively. For each gene, all samples were amplified simultaneously in duplicate in one assay run. Data were analyzed using the comparative cycle threshold (Ct) method. The target RNA level was normalized to GAPDH RNA level as previously described [28]. A blank sample containing water, primers and Eva Green but no template was also included.

Western blotting analysis
MDA-MB-231 cells were seeded (10⁵ cells/well) in a six-well plate in culture medium (DMEM) plus 10% FBS overnight at 37 °C and 5% CO₂ and then incubated with 10, 100 or 1000 nM ALT-C. After 24 h, culture medium was removed and cells were lysed with RIPA buffer [150 mM NaCl; 50 mM Tris; pH 8.0; 0.1% sodium dodecyl sulfate (SDS); 1% Triton X-100] and proteases and phosphatases inhibitors. Protein quantitation was carried out using the BCA Protein Assay kit (Thermo Scientific, USA), according to the supplier’s instructions.

Thirty micrograms of each sample were diluted in denaturing sample buffer containing glycerol, SDS, dithiothreitol (DTT) and bromophenol blue. After electrophoresis, the samples were transferred to nitrocellulose membranes and blocked with skimmed milk powder (4%). Rabbit monoclonal antibodies against p-FAK (ab81298) and FAK (ab40794) were used at 1:1000 dilution in PBS. A secondary anti-rabbit antibody (ab97051) was used at 1:10,000 dilution in milk powder. Detection of proteins was performed using the Chemiluminescent Peroxidase Substrate-1 (SLB1875, Sigma-Aldrich, USA). Images were obtained on a digital documentation system (Chemi-Doc Xr, Bio-Rad Lab, USA) and relative quantitation was done by densitometric analysis of the images using the Image J software and normalizing to GAPDH band densities when indicated.

Statistical analysis
Each experiment was repeated twice (n = 2) in triplicate and the mean and standard errors were calculated. The results were compared statistically using one-way analysis of variance (ANOVA) and Tukey’s test was used when p values were *p < 0.05, **p < 0.01 or ***p < 0.001. Statistical comparisons were done in relation to the 0 nM condition.

Results
ALT-C affinity to α₂β₁ integrin was higher than to collagen I
Our group has previously demonstrated the binding of ALT-C to α₂β₁ integrin by inhibition of cell adhesion assays in Col I coating; however, the kinetics parameters of this association were never determined. In addition, it has been suggested that ECD-proteins bind directly to collagen as well as to α₂β₁ integrin, which could have implications in their mechanism of action. To address the question if ALT-C could bind to Col I and to α₂β₁ integrin, we determined the ALT-C dissociation constant (K_d) values for α₂β₁ integrin and Col I by surface plasmon resonance (SPR). α₂β₁ integrin and Col I were immobilized to a carboxymethylated dextran (CM5) sensor chip and ALT-C was passed over the layers in PBS buffer. ALT-C bound to α₂β₁ integrin with a K_d ≈ 1.4 μM (Fig. 1a); in contrast, a lower affinity was found for Col I, with a K_d ≈ 48 μM (Fig. 1b). ALT-C binding to α₂β₁ integrin was approximately 35 times stronger than to Col I.

The SPR was also performed for ALT-C binding to α₃β₃, α₂β₃ and fibronectin (FN). As expected, ALT-C did not bind to α₃β₃ and α₂β₃ integrins or to FN, confirming the specificity of ALT-C for α₂β₁ and Col I (Additional file 1).

Characterization of integrin cell expression by flow cytometry
The expression of the α₂β₁ integrin subunits on cell surfaces was analyzed by flow cytometry. The three cell lines used in this work have similar expression profiles of α₂β₁ integrin with high contents of α₂ and β₁ integrin subunits (Additional file 2). Therefore, these cells were considered comparable models to investigate the role of α₂β₁ integrin on MMPs and cell migration. ALT-C treatment (10–1000 nM) did not change the α₂ subunit
Fig. 1 Characteristics of ALT-C binding to $\alpha_2\beta_1$ integrin or collagen type I (Col I). Representative sensorgrams and dose-dependent binding of ALT-C (0.0–5.0 $\mu$M) measured by surface plasmon resonance (SPR): (a) to $\alpha_2\beta_1$ integrin; and (b) to Col I. Kinetic curves were analyzed using the 1:1 binding model by GraFit 7 software for: (c) $\alpha_2\beta_1$ integrin; and (d) Col I.
content of human breast adenocarcinoma cells (MDA-MB-231), which was also confirmed by Western blotting (Additional file 3).

ALT-C increased MDA-MB-231 cell trans-endothelial migration
Since disintegrins and disintegrin-like proteins are known to competitively inhibit cell migration, we first investigated whether α2β1 integrin could be a relevant player for tumor cell transmigration using ALT-C as a α2β1 integrin ligand. ALT-C increased tumor cell transmigration through a layer of endothelial cells at concentrations of 10 and 40 nM by 67.9% and 116.5%, respectively (Fig. 2a). From a concentration of 100 nM and higher, ALT-C lost this ability. Tumor cells were labeled using the PKH26 Red Fluorescent Cell Linker kit for General Cell Membrane Labeling® (Sigma-Aldrich, USA) to distinguish them from HMEC-1 cells. All cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Fig. 2b).

ALT-C inhibited MMPs in the conditioned media of cell cultures from a wound healing assay
To study the role of α2β1 integrin in MMP content, we tested ALT-C in a wound healing assay, which is another commonly used migration assay. No ALT-C effect was observed on the migration ability of MDA-MB-231 cells (Fig. 3a), fibroblasts (Fig. 3b) or HMEC-1 cells (Fig. 3c) in wound healing assays. These results suggest that the ALT-C effects may be depend on one or more FBS components to stimulate migration, since ALT-C acts on cell migration through endothelial cells in presence of FBS (Fig. 2). The total content of MMP-9 and MMP-2 in the conditioned media from ALT-C-treated cells was analyzed by gelatin zymography after the wound healing assay. ALT-C significantly decreased MMP-9 content in the conditioned media from MDA-MB-231 cell culture at all concentrations used (Fig. 4a). MMP-2 content was also decreased in MDA-MB-231 cells but only at the 100 and 1000 nM concentrations of ALT-C. MMP content was not changed in the conditioned media from human fibroblast culture treated with ALT-C (Fig. 4b). All concentrations of ALT-C (10, 40, 100 and 1000 nM) significantly decreased MMP-2 level in HMEC-1 cells (Fig. 4c). MMP-9 was not detected in the conditioned media of fibroblasts or HMEC-1 cells.

ALT-C induced the increase of c-Myc mRNA level in MDA-MB-231 cells
The oncogene c-Myc has been associated with the expression of integrin genes in cells from different tissues and its overexpression significantly inhibited the migration and invasiveness of MDA-MB-231 cells in vitro [29]. Therefore, we investigated whether ALT-C could trigger the increase of c-Myc mRNA levels upon α2β1 integrin binding. ALT-C upregulated c-Myc mRNA level at 1000 nM in MDA-MB-231 cells (Fig. 5d). However, no effect on c-Myc mRNA level was observed in human fibroblasts (Fig. 5e). Conversely, ALT-C inhibited c-Myc mRNA level in HMEC-1 cells at 10 nM (Fig. 5f).

A low concentration of ALT-C induced FAK phosphorylation in MDA-MB-231 cells
To address whether ALT-C was activating α2β1 integrin we sought to determine the content of phosphorylated
FAK (p-FAK), which is a hallmark of integrin activation. After 24 h of incubation, ALT-C resulted in highly phosphorylated FAK at 10 nM, but not at 100 or 1000 nM in MDA-MB-231 cells (Fig. 6).

**Discussion**

Previous studies from our group have shown that ALT-C specifically inhibits the adhesion of α2β1-overexpressing human chronic myelogenous leukemia cells (K562) to collagen I with a half-maximal inhibitory concentration (IC50) of 100 nM [24]. Later, it was reported that jararhagin-C, an ALT-C homolog from *Bothrops jararaca* venom, also binds directly to collagen through its disintegrin domain [30]. However, the relevance of this collagen-binding property to the mechanism of action of such proteins was not well understood. To further elucidate this mechanism, we determined the Kd of ALT-C to both collagen and its receptor by plasmon resonance. Our data corroborate those of Souza et al. [24], confirming the strong interaction between ALT-C and α2β1 integrin (Kd ≈ 1.4 μM). ALT-C affinity to collagen was much lower (Kd ≈ 48 μM). Therefore, it was demonstrated that the predominant target of ALT-C is α2β1 integrin. Using the same approach, we have recently reported that a disintegrin from *Bothrops alternatus* (DisBa-01), a recombinant RGD-disintegrin from *Bothrops alternatus*, binds to α5β1 and α6β1 integrins with high affinity (Kd = 4.63 × 10^{-7} and 7.62 × 10^{-5} M, respectively) [31]. Therefore, the affinity of α2β1 integrin for ALT-C is approximately five times higher than of α5β1 integrin for an RGD disintegrin.

ALT-C in low concentrations increased the transmigration of MDA-MB-231 cells through an endothelial cell layer. This assay is intended to simulate the extravasation of tumor cells through blood vessels that leads the establishment of metastasis [17]. Previous results demonstrated a potent chemotactic effect of ALT-C on neutrophils, an effect mediated by FAK and PI3K activation [32]. ALT-C also increased protein kinase B (Akt/PKB) phosphorylation in endothelial cells, which is a key signaling pathway for cell survival [25]. However, higher ALT-C concentrations did not produce the same chemotactic effect. This bell-shape result of the concentration-effect curve was previously observed in our first study with ALT-C [33], and might be due to receptor internalization. Higher concentrations of ALT-C also inhibited
FAK phosphorylation, in agreement with the transmigration results. The activation of the FAK/PI3K/Akt axis results in the phosphorylation of several proteins involved in the polymerization and stabilization of the actin cytoskeleton that are necessary for cell migration [34, 35]. Our data suggest that, at low concentrations, ALT-C binding to \( \alpha_2\beta_1 \) integrin triggers integrin-mediated intracellular signaling events such as FAK phosphorylation and stimulation of cell transmigration. However, at higher concentrations, FAK is not activated, and the tumor cells lose the ability to transmigrate.

We did not observe any effects of ALT-C in the wound healing assay, and then we tested the MMP-2 and MMP-9 contents in the conditioned media from these experiments. We observed that ALT-C decreased the content of both enzymes in the supernatants of the MDA-MB-231 cultures, and that of MMP-2 in the HMEC cultures, without any effect on the MMP content on the fibroblast cultures. These results suggest that wound closure in this assay does not depend on MMPs. We have not analyzed MMP content in the supernatants from the transmigration assays due to the presence of serum in the media, which causes strong interference in the zymographic analysis. However, the possibility that cells are transmigrating in an MMP-independent way should be considered as previously suggested by others [36, 37].

MMPs can be regulated by integrins through different pathways related to ECM remodeling. A study showed that function-blocking anti-\( \alpha_3 \) antibodies decrease MMP-9 activity in MDA-MB-231 cells [38]. The \( \alpha_2\beta_1 \) integrin seems to regulate the selection of a specific polyadenylation site within the MMP-9 mRNA via the activation of an integrin-mediated extracellular signal-regulated kinase (MEK/ERK) signaling pathway, resulting in the generation of a short and more stable transcript and the subsequent synthesis of MMP-9 protein [39]. When the integrin is blocked or silenced, MEK/ERK signaling is decreased and a longer mRNA is produced that is more easily subjected to degradation without the generation of MMP-9 protein. We believe that a similar mechanism would also be possible for \( \alpha_2\beta_1 \) integrin, although this hypothesis has not been confirmed.

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**Fig. 4** Effects of ALT-C on the MMP-9 and MMP-2 contents in the conditioned media from (a) MDA-MB-231, (b) human fibroblasts, and (c) HMEC-1 cells. MMP content was detected by band densitometry in the conditioned media (peak area) after wounding and incubation with ALT-C for 24 h. MMP-2 and MMP-9 contents were determined by band densitometry. The assay was performed in triplicate with two independent assays (n = 2). * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) compared to control (without ALT-C).
Fig. 5  (a) ALT-C effects on MMP-9 expression in MDA-MB-231 cells, and (b) on MMP-2 mRNA levels in fibroblasts or (c) HMEC-1 cells. Levels of c-Myc mRNA after ALT-C treatment in (d) MDA-MB-231 cells, (e) human fibroblasts, and (f) HMEC-1 cells. The values represent relative transcript abundance and the p value was determined using ANOVA followed by Tukey’s test. Values were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. The assay was performed in triplicate with two independent assays (n = 2). * p < 0.05, *** p < 0.001 compared to control (0 nM).

Fig. 6 ALT-C induces FAK phosphorylation at 10 nM but not at 100 and 1000 nM.  (a) FAK and p-FAK protein level revealed by Western blotting in lysates extracted from MDA-MB-231 treated with ALT-C (10, 100 and 1000 nM) and control (0 nM).  (b) Values represent the normalized densitometry ratio of p-FAK and FAK and the p value was determined using ANOVA followed by Tukey’s test. Values were also previously normalized to the level of GAPDH densitometry. ** p < 0.01 compared to control (0 nM). The assay was performed in independent assays (n = 2) and Western blotting in quadruplicate.
For tumor growth, new vessels are necessary to maintain its nutrition. The process of tumor angiogenesis involves several steps of cell–cell and cell–matrix interactions that allow endothelial cells to migrate towards the tumor. Proteases are also required to cleave the basement membrane and interstitial matrix molecules, including MMPs [40]. Among the MMPs, MMP-2 has been described as playing a key role in angiogenesis and in the invasiveness phenotype as well [40–42]. We have previously demonstrated that ALT-C modulates fibroblast growth factor (FGF)-induced angiogenesis in vivo using the Matrigel plug model in nude mice [33]. In that study, we observed that low concentrations of ALT-C are pro-angiogenic, but high concentrations such as 1000 nM, completely inhibited angiogenesis. In the present study, we demonstrated that ALT-C decreased MMP-2 protein level in endothelial cells at all tested concentrations, which could be prejudicial to the formation of new vessels since MMP-2 is required for angiogenesis. Our data corroborate other studies in which MMP-2 downregulation in cancer cells and Mmp2-deficient mice show reduced angiogenesis and tumor growth [43]. It is possible that ALT-C is interfering with the mechanisms underlying MMP-2 activation, but additional assays are required to address this question.

Oncogenes such as c-Myc are involved in metastasis by affecting a number of cellular processes, including cell growth, proliferation and apoptosis [44, 45]. Paradoxically, studies have demonstrated that c-Myc can also act as a tumor suppressor and is related to integrins in cell adhesion and migration [29]. Recently, it was reported that αβ1 collagen receptor expression is controlled by c-Myc in colorectal cancer cells [46]. However, the mechanisms underlying the control of c-Myc RNA level are not well understood. Some studies have demonstrated that c-Myc transcription is related to an AKT-dependent mechanism [47, 48], while others have shown that FAK is associated with the PI3 kinase/AKT pathway in tumor progression [49, 50]. More recently, it was reported that PI3 Kinase/AKT signaling promotes c-Myc activation [51].

Previous results demonstrated the relation between ALT-C binding to αβ1 integrin and the activation of the PI3 kinase/AKT axis and activation of FAK as well [25, 32]. ALT-C (1000 nM) upregulated c-Myc mRNA level in MDA-MB-231 cells, but not in fibroblasts. Surprisingly, c-Myc mRNA level in endothelial cells was downregulated by 10 nM of ALT-C, but not by higher concentrations. c-Myc is considered an oncogene, and the overexpression of c-Myc significantly inhibited migration and reduced the invasiveness of MDA-MB-231 cells in vitro [29]. On the other hand, Magid et al. [52] suggested that c-Myc activates MMP-9 RNA level in endothelial cells under shear stress. Since c-Myc is related to the transcription of integrin genes in cells from different tissues [29, 53], we hypothesize that ALT-C binds to and activates αβ1 integrin upregulating c-Myc mRNA level via activation of the FAK/PI3K/AKT axis.

Studies have shown that the overexpression of c-Myc and of αβ1 integrin reduces invasion and metastasis in MDA-MB-231 breast tumor cells [17, 29]. Together, these results indicate a role for αβ1 integrin in c-Myc activation and tumor progression. Thus, Fig. 7 provides the potential ALT-C mechanisms. To the best of our knowledge, this is the first report of c-Myc upregulation by αβ1 integrin activation after ligand binding.

Conclusions
In summary, these results suggest that ALT-C binds to αβ1 integrin in tumor cells and inhibits MMP-9 and MMP-2, but upregulates c-Myc (mRNA level). In endothelial cells, ALT-C decreases MMP-2 content required for angiogenesis as demonstrated by zymography. Fibroblasts are insensitive to this integrin, at least regarding the activities studied in this work. Based on the present study, we propose that ALT-C interferes with the tumor progression by binding to the αβ1 integrin tumor cells. It is also hypothesized that ALT-C impairs angiogenesis by reducing MMP-2 content in endothelial cells. All together, these results highlight the possibilities of interfering in the tumor microenvironment and consequently in tumor progression by considering αβ1 integrin as a target against metastasis.
The authors declare that they have no competing interests.

Competing interests
Not applicable.

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Authors’ contributions
HSSA conceived and designed the experiments. MNOM, LMSE, KCM and TCV carried out the experiments. PKS and ACCN were in charge of the SPR analysis (UNIFESP, São Paulo). Thanks are also due to Marcelo Ducatti Aparecida Sadae Tanaka and the technician Ricardo José Soares Torquato for the graphic art.

Ethics approval
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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