

RESEARCH ARTICLE

Retinoic acid receptor gamma impacts cellular adhesion, Alpha5Beta1 integrin expression and proliferation in K562 cells

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Abstract

The interplay between cellular adhesion and proliferation is complex; however, integrins, particularly the $\alpha 5 \beta 1$ subset, play a pivotal role in orchestrating critical cellular signals that culminate in cellular adhesion and growth. Retinoids modify the expression of a variety of adhesive/proliferative signaling proteins including $\alpha 5 \beta 1$ integrins; however, the role of specific retinoic acid receptors involved in these processes has not been elucidated. In this study, the effect of all-*trans*-retinoic acid receptor (RAR) agonists on K562 cellular adhesion, proliferation, and $\alpha 5 \beta 1$ integrin cell surface expression was investigated. RAR γ agonist exposure increased K562 cellular adhesion to RGD containing extracellular matrix proteins fibronectin and FN-120 in a time- and concentration dependent manner, while RAR α or RAR β agonist treatment had no effect on cellular adhesion. Due to the novel RAR γ - dependent cellular adhesion response exhibited by K562 cells, we examined $\alpha 5$ and $\beta 1$ integrin subunit expression when K562 cells were exposed to retinoid agonists or vehicle for 24, 48, 72 or 96 hours. Our data demonstrates no differences in K562 cell surface expression of the $\alpha 5$ integrin subunit when cells were exposed to RAR α , RAR β , or RAR γ agonists for all time points tested. In contrast, RAR γ agonist exposure resulted in an increase in cell surface $\beta 1$ integrin subunit expression within 48 hours that was sustained at 72 and 96 hours. Finally, we demonstrate that while exposure to RAR α or RAR β agonists have no effect on K562 cellular proliferation, the RAR γ agonist significantly dampens K562 cellular proliferation levels in a time- and concentration- dependent manner. Our study is the first to report that treatment with a RAR γ specific agonist augments cellular adhesion to $\alpha 5 \beta 1$ integrin substrates, increases cell surface levels of the $\beta 1$ integrin subunit, and dampens cellular proliferation in a time and concentration dependent manner in a human erythroleukemia cell line.

Introduction

Retinoids, vitamin A and its analogs, play a critical role in modulating the precise balance between cellular adhesion and proliferation that is required for maintenance of proper cell homeostasis [1–7]. Biologically active metabolites of vitamin A, all-*trans*-retinoic acid and 9-

cis-retinoic acid are reported to mediate a number of cellular functions including differentiation, proliferation, adhesion, transmigration and more recently integrin expression [8–10].

All-*trans*-retinoic acid (*t*-RA) and 9-*cis*-retinoic acid (9-*cis*-RA) activate retinoic acid receptors (RAR α , β , and γ) and retinoid X receptors (RXR α , β , and γ). These retinoids act as ligand-dependent transcription factors with *t*-RA activating RARs, while 9-*cis*-RA serves as a pan-agonist for RARs and RXRs [11–15]. These receptor complexes act as heterodimers or homodimers binding to specific retinoid response elements, RARE and RXRE, in the promoter of target genes [16]. These distinct retinoid receptor partnerships differentially regulate transcription of a number of targets who actively participate in the complex interplay of cellular adhesion and proliferation, including integrins, and their extracellular matrix counter-receptors [9, 17–20].

Integrin-mediated adhesion to the extracellular matrix stringently regulates cell cycle progression [21, 22]. Integrins, a family of transmembrane heterodimeric receptors consisting of non-covalently linked α and β subunits are the principle receptors involved in attachment to the extracellular matrix. The $\alpha 5\beta 1$ integrins are fibronectin (FN) receptors with the RGD sequence in the FNIII domain 10 being the crucial attachment site for $\alpha 5\beta 1$ [23]. Signaling processes that are crucial for regulating cell proliferation follows initial $\alpha 5\beta 1$ integrin binding of the RGD sites in FN [24–28]. There have been a number of studies specifically demonstrating that altered $\alpha 5\beta 1$ integrin expression results in modulated cellular proliferation [29–32]. Interestingly, retinoids have been implicated in altering integrins, including $\alpha 5\beta 1$ [18, 20].

Although the ability of *t*-RA and 9-*cis*-RA to alter cell adhesion and proliferation is addressed in the literature, there is a shortage of information regarding which retinoid receptors govern these particular cellular functions through modulation of the $\alpha 5\beta 1$ integrin subset. We present evidence that exposure to a RAR γ agonist augments adhesion to both fibronectin and the $\alpha 5\beta 1$ specific chymotryptic fragment of fibronectin (FN-120) in a time and concentration dependent manner. Further, we investigated the effects of retinoid agonists on both the integrin $\alpha 5$ and $\beta 1$ subunit cell surface expression in K562 cells and report that the $\beta 1$ integrin subunit is increased on the cell surface in response to RAR γ agonist treatment. Finally, we demonstrate that the RAR γ agonist dampens cellular proliferation in a time and concentration dependent manner. Our study is the first to report that a RAR γ agonist augments cellular adhesion, dampens cellular proliferation, and increases $\beta 1$ integrin cell surface expression in a human erythroleukemia cell line.

Materials and methods

Reagents and chemicals

Human fibronectin (FN) was purchased from BD Biosciences (Bedford, MA). The purified human fibronectin alpha-chymotryptic fragment, FN-120, was purchased from Millipore (Temecula, CA). Anti-integrin $\alpha 5$ FITC conjugated antibody (SAM-1), and MOPC-21 (isotype control) was purchased from Abcam (Cambridge, MA). The anti-integrin $\beta 1$ FITC conjugated antibody was purchased from Millipore. Retinoid agonists RAR α (AM 580) RAR β (AC 55649) and RAR γ (CD 437) were purchased from Tocris (Minneapolis, MN). Agonists were stored at -20°C or $+4^{\circ}\text{C}$. Retinoids were dissolved at the desired concentration in ethanol. The compound *p*-nitro-phenyl phosphate was purchased from Sigma (St. Louis, MO).

Cell culture

The human cell line K562 was obtained from ATCC (Manassas, VA) and maintained in RPMI 1640 supplemented with 10 mM HEPES, 1mM sodium pyruvate, 10% (v/v) fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin at 37°C in an atmosphere of 5% CO_2 .

Cellular adhesion assays

Static adhesion assays to immobilized ligands were adapted from established techniques [33, 34]. Briefly, fibronectin, or FN-120 were immobilized at desired concentrations on 96-well Immulon-2 HB microtiter plates (Thermo Scientific, Waltham, MA) in a total volume of 100 μ l of 0.1 M NaHCO₃ pH 8.4 overnight at 4°C. Nonspecific adhesion was minimized by blocking wells with 2% (w/v) bovine serum albumin (BSA) in 0.1 M NaHCO₃ at room temperature for 1 hr. K562 cells were cultured for the designated times (24, 48, 72, or 96 hrs) in the presence of RAR α agonist (AM 580) (1 μ M), RAR β agonist (AC 55649) (1 μ M), RAR γ agonist (CD 437) (0.125–1 μ M), or an equimolar concentration of ethanol. Before addition to wells, cells were washed twice in HEPES-Tyrodes buffer, enumerated, and added to wells (9×10^4 cells/well) in HEPES-Tyrodes with 1 mM MnCl₂. Cells were incubated in the 30 min (fibronectin ligand) or 1 hr (FN-120 ligand) at 37°C in 5% CO₂. After three consecutive washes with HEPES-Tyrodes, wells were analyzed for bound cells by determining the relative cellular acid phosphatase activity within each well. Phosphatase assay buffer (1% v/v Triton X-100, 50 mM sodium acetate at pH 5.0 and 6 mg/mL *p*-nitrophenyl phosphate) was added to wells, and wells were incubated with the substrate for 30 minutes at 37°C. Color was disclosed by addition of 50 μ l/well of 1 N NaOH. Absorbance values were obtained at 405 nm using a Biotek microplate reader. Adhesion values obtained with wells coated exclusively with BSA were considered as background values for each experimental condition and were subtracted before reporting final values. Adhesion values were normalized using Percent Vehicle = (Abs_(treatment)/Abs_(vehicle)) X 100. Results are shown as means \pm SD, n = 6.

Flow cytometry

Cells were treated with 1 μ M RAR α agonist (AM 580), RAR β agonist (AC 55649), RAR γ agonist (CD 437), or an equimolar concentration of ethanol for designated time periods (24–96 hrs). Cells were harvested and washed twice in PBS containing 3.0% (w/v) BSA (PBS-BSA). 1.0×10^6 cells were re-suspended in a total volume of 1 mL PBS-BSA, anti- α 5 integrin antibody, anti- β 1-integrin antibody, or isotype control (10 μ g/mL) was added, and cells were incubated on ice for 30 min. Unbound antibody was removed by three final washes in PBS-BSA, and fluorescent intensity was analyzed on Beckman-Coulter Quanta SC flow cytometer (Beckman-Coulter, Inc. Brea, CA).

BrdU cell proliferation assay

The ELISA-based BrdU cell proliferation kit was purchased from Chemicon International (Temecula, CA). Cells were plated at 2.5×10^4 cells/mL in 100 μ l culture media containing RAR α agonist (AM 580) (1 μ M), RAR β agonist (AC 55649) (1 μ M), RAR γ agonist (CD 437) (0.125–1 μ M), or an equimolar concentration of ethanol. K562 cells were cultured for a total of 24–96 hrs at 37°C at 5% CO₂ atmosphere. All treatments were diluted to their final concentrations with media to desired concentrations to avoid proliferation effects due to high ethanol concentrations. Absorbance values were obtained with a microplate reader at 450 nm. BrdU incorporation was assessed by ELISA and proliferation values were normalized using Percent Vehicle = (Abs_(treatment)/Abs_(vehicle)) X 100. Results are shown as means \pm SD, n = 6.

Statistical analysis

LSD was used to test for differences among groups (P = 0.01)

Results

RAR γ agonist exposure augments K562 cellular adhesion to RGD containing extracellular matrix proteins in a time-dependent manner

Retinoids have been established as having a role in governing integrin-dependent cellular adhesion [18, 35]. In K562 cells, retinoids have been demonstrated to restore cellular adhesion to RGD substrates [18]. However, there is paucity amount of information regarding which retinoid receptors govern cellular adhesion, particularly in K562 cells. To evaluate if retinoid receptor agonists alters cellular adhesion in K562 cells, we employed a static cell adhesion assay utilizing fibronectin (FN) or FN-120, which were selected due to their well-defined RGD binding sites. As shown in Fig 1A, K562 cells treated with 1 μ M RAR α or RAR β agonists for 72 hours had similar levels of cellular adhesion on fibronectin at 5 μ g/mL as compared to vehicle. Conversely, RAR γ agonist treated cells had a 44% increase in adhesion compared to vehicle

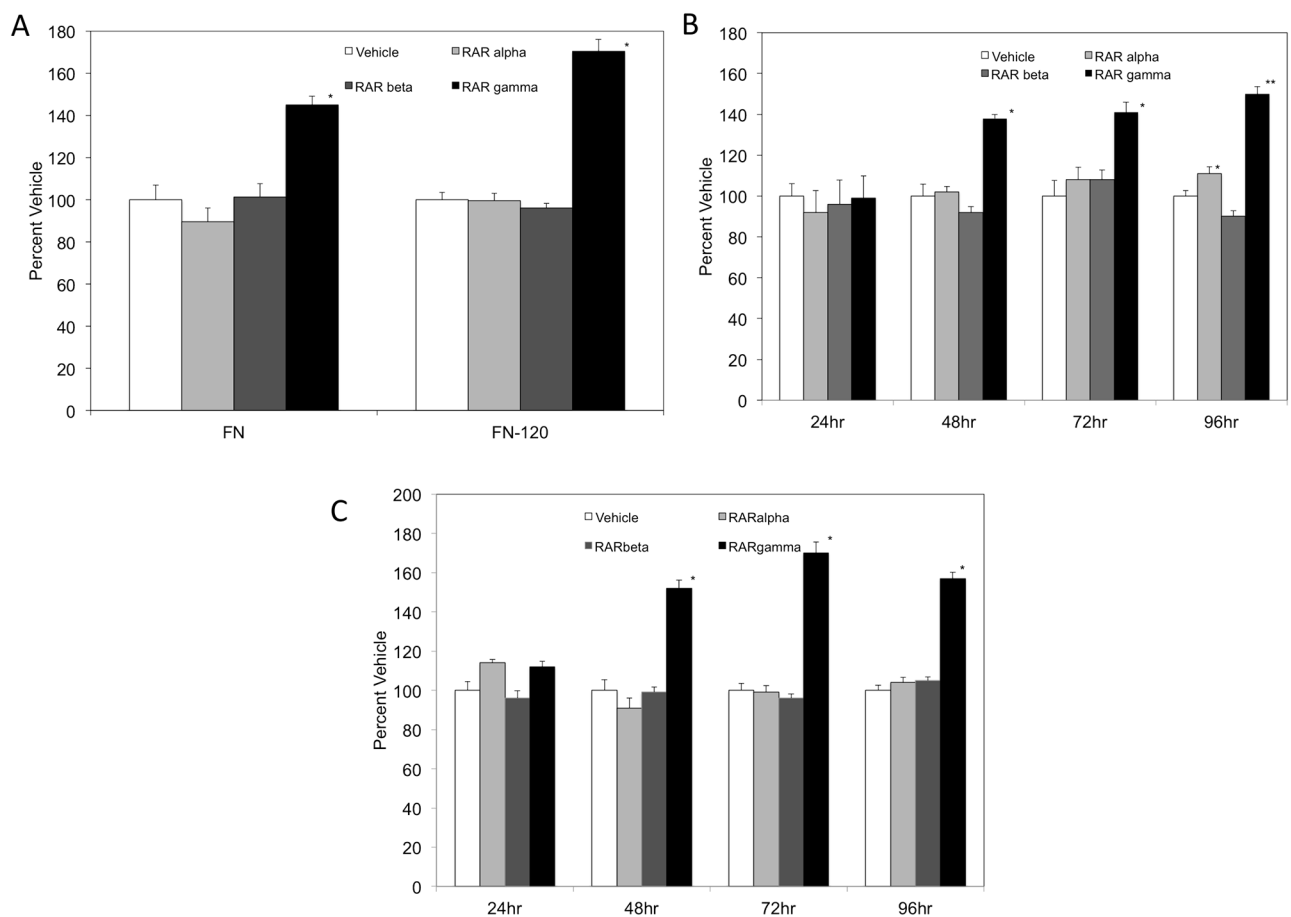


Fig 1. K562 cellular adhesion to fibronectin and FN-120 is increased in a time-dependent manner in the presence of an RAR γ agonist. (A) Microtiter wells were coated with 5 μ g/mL fibronectin or FN-120. K562 cells cultured for 72 hrs in the presence of vehicle (white bars), 1 μ M RAR α agonist (light gray bars), RAR β agonist (dark gray bars) or RAR γ agonist (black bars) were added to wells (9×10^4 cells/well) in HEPES-Tyroses buffer containing 1 mM MnCl₂. (B) K562 cells cultured for 24, 48, 72, or 96 hrs in the presence of vehicle (white bars), 1 μ M RAR α agonist (light gray bars), RAR β agonist (dark gray bars) or RAR γ agonist (black bars) were added to wells coated with 5 μ g/mL fibronectin, and adhesion assays were repeated. (C) K562 cells cultured for 24, 48, 72, or 96 hrs with vehicle (white bars), 1 μ M RAR α agonist (light gray bars), RAR β agonist (dark gray bars) or RAR γ agonist (black bars) were added to wells coated with 5 μ g/mL FN-120, and adhesion assays were repeated. Percent Vehicle = $\frac{Abs_{405nm}(treatment)}{Abs_{405nm}(vehicle)} \times 100$. Results are expressed as means \pm SD, n = 6, LSD was used to test for differences among groups. Means followed by * or ** are significantly different (P = 0.01).

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treatment on the fibronectin ligand. Fibronectin contains binding sites for a number of integrins, including the $\alpha 5\beta 1$ subset. To determine the contribution to cellular adhesion by only the $\alpha 5\beta 1$ integrin subset, the 120-kDa chymotryptic fragment with the RGD recognition sequence (FN-120) was utilized and cellular adhesion assays were repeated (Fig 1A). RAR α and RAR β agonists treated cells had cellular adhesion values comparable to the vehicle. Interestingly, on the FN-120 substrate, RAR γ agonist treated cells had a 70% increase in cellular adhesion when compared vehicle treated cells.

To address if the observed augmented adhesion was time-dependent, we restricted the exposure of K562 cells to these agonists for 24, 48, 72 or 96 hrs. When cells were added to wells coated with FN, similar levels of cellular adhesion were obtained for all treatment groups cultured for 24 hours (Fig 1B). At 48 hours, a significant increase in cellular adhesion was observed in K562 cells treated with the RAR γ agonist on the FN ligand. However, there were no statistical differences between cellular adhesion levels of K562 cells treated with vehicle, RAR α agonist, or RAR β agonists for 48 hours. At 72 hours, K562 cells exposed to the RAR γ agonist obtained statistically higher cellular adhesion levels with an increase of 41% in cellular adhesion compared to vehicle treatment only on the FN substrate. K562 cells cultured with RAR α or RAR β agonists for 72 hours had comparable levels of cellular adhesion to vehicle treatment alone. At 96 hours, significant levels of cellular adhesion were observed with cells dosed with the RAR γ agonist with a 50% increase in cellular adhesion compared to vehicle control.

As shown in Fig 1C, a similar trend in cellular adhesion was observed when cells were added to wells coated with FN-120. K562 cells cultured in the presence of retinoid receptor agonists for 24 hours had no statistical differences between cellular adhesion levels as compared to vehicle control. At 48 hours, a significant increase in cellular adhesion was observed when cells were treated with the RAR γ agonist utilizing the FN-120 ligand. There were no statistical differences between cellular adhesion levels of K562 cells treated with vehicle, RAR α agonist, or RAR β agonists for 48 hours. At 72 hours, K562 cells exposed to the RAR γ agonist had a robust 70% increase in cellular adhesion compared to vehicle treatment only. Cells dosed with RAR α or RAR β agonists had comparable levels of cellular adhesion to vehicle treatment alone. At 96 hours, a significant increase in cellular adhesion (57%) was observed when cells were dosed with the RAR γ agonist compared to vehicle control. However, there were no statistical differences between cellular adhesion levels of K562 cells treated with vehicle, RAR α agonist, or the RAR β agonist at 96 hours. Collectively, our data demonstrates that treatment with the RAR γ agonist results in a robust increase in cellular adhesion that is time dependent with significant augmented cellular adhesion observed within 48 hours of RAR γ agonist treatment.

RAR γ agonist increases K562 cellular adhesion to FN and FN-120 in a concentration-dependent manner

Retinoids have been evaluated for their therapeutic potential; however, their use has been limited due to toxicity and teratogenic side effects [1]. Since the RAR γ agonist increases cellular adhesion to both fibronectin and FN-120 at 1 μ M, we evaluated whether lower concentrations of the RAR gamma agonist confers cellular adhesion to these ligands. The agonist concentration chosen were based on reported EC₅₀ values [36]. As shown in Fig 2, K562 cells cultured in the presence of the RAR γ agonist had increased cellular adhesion to FN starting at a concentration of 500 nM. When cells were dosed with RAR γ agonist at 500 nM or 1 μ M, cellular adhesion was increased by 24% and 44%, respectively. Cellular adhesion values were comparable to the vehicle when K562 cells were dosed with 125 nM or 250 nM RAR γ agonist. Interestingly,

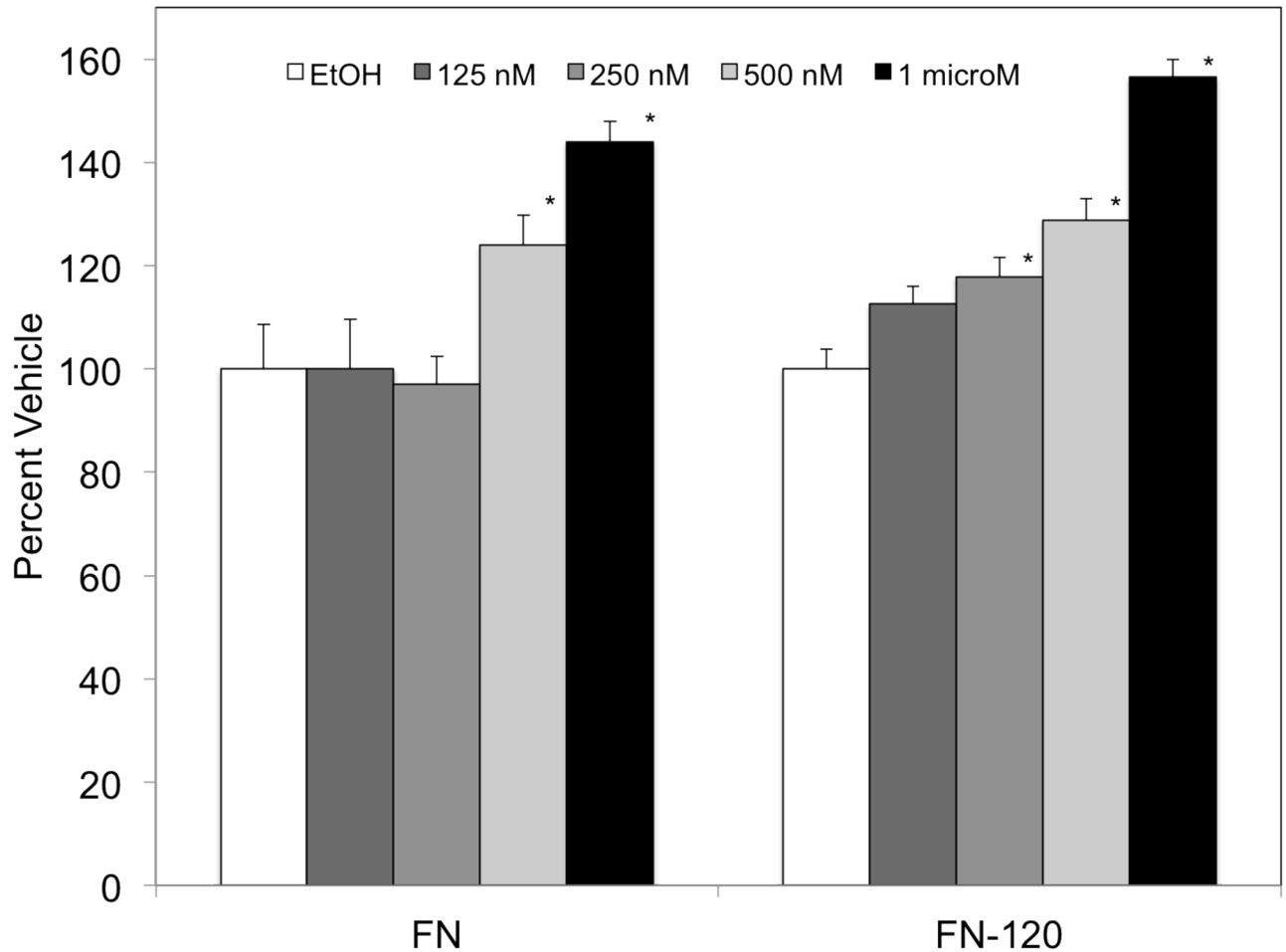


Fig 2. RAR γ agonist exposure augments K562 cellular adhesion to RGD containing extracellular matrix proteins in a concentration dependent manner. (A) K562 cells were cultured for 72 hrs in the presence of vehicle (white bars), 125 nM RAR γ agonist (dark gray bars), 250 nM RAR γ agonist (medium gray bars), 500 nM RAR γ agonist (light gray bars) or 1 μ M RAR γ agonist (black bars) After 72 hrs of treatment, K562 cells were added to wells (9×10^4 cells/well) in Heps-Tyroses buffer containing 1 mM MnCl₂ to wells coated with 5 μ g/mL of fibronectin, or FN-120. Percent Vehicle = $\text{Abs}_{405\text{nm}}(\text{treatment}) / \text{Abs}_{405\text{nm}}(\text{vehicle}) \times 100$. Results are expressed as means \pm SD, n = 6, LSD was used to test for differences among groups. Means followed by * are significantly different (P = 0.01).

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when the FN-120 ligand was utilized a statistically significant increase in cellular adhesion was observed at the lowest RAR γ agonist concentration of 125 nM. As shown in Fig 2, K562 cells cultured in the presence of 125 nM, 250 nM, 500 nM or 1 μ M RAR γ agonist had continual increasing levels of adhesion to FN-120 ligand.

RAR γ agonist augments cell surface expression of the β 1 integrin subunit in a time-dependent manner

Retinoids have been shown to modulate the integrin repertoire expressed on the cell surface of a number of cell lines, including K562 cells [17, 18, 35]. A number of integrin subunits have been profiled on the K562 cell surface in the presence of 9-*cis*-RA and recent studies have shown that retinoids are capable of impacting only the α 5 β 1 subset on K562 cells [18]. Due to the novel adhesion response exhibited by K562 cells, we examined integrin expression of α 5 and β 1 subunits when K562 cells were exposed to 1 μ M RAR α , β , or γ agonists or equimolar concentration of vehicle for 24, 48, 72 or 96 hours. Utilizing the anti- α 5 integrin antibody, our

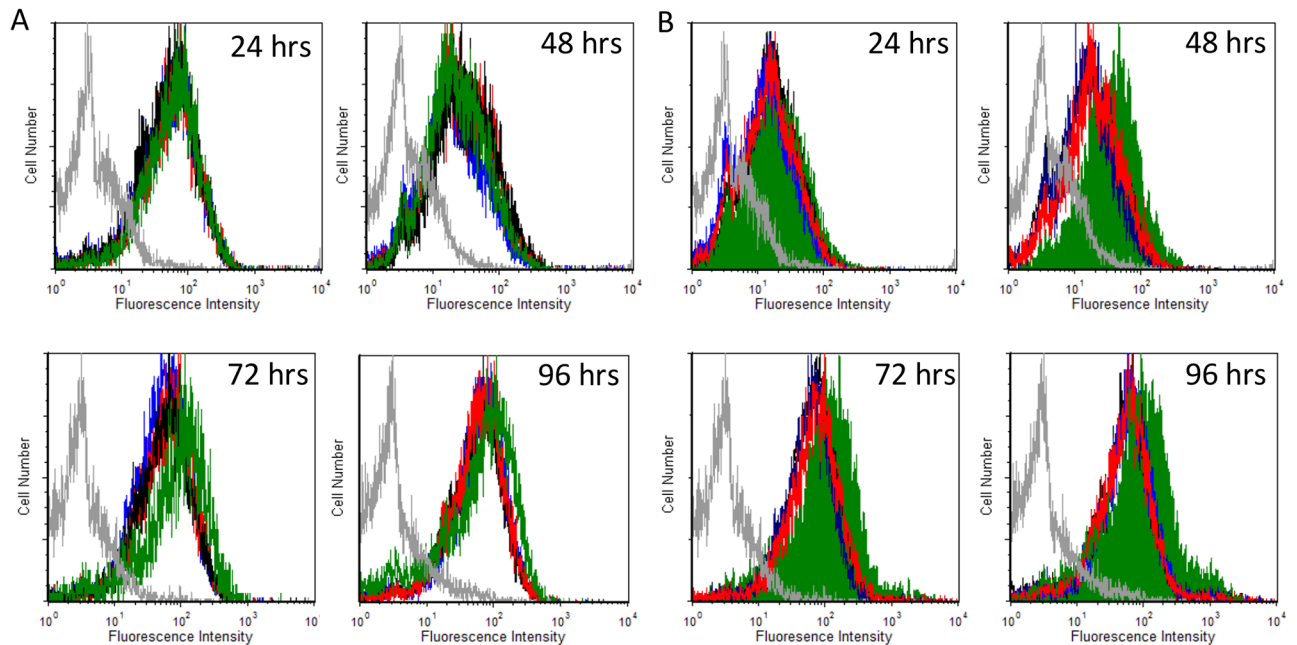


Fig 3. An RAR agonist augments the cell surface expression of the $\beta 1$ integrin subunit in a time-dependent manner. (A) The extent of mAb SAM-1 (anti- $\alpha 5$) binding to K562 cells exposed to vehicle, 1 μ M RAR α agonist, RAR β agonist, or RAR γ agonist for 24, 48, 72, or 96 hrs was assessed by FACS analysis. SAM-1 was used at a concentration of 10 μ g/mL. Cell surface levels of the $\alpha 5$ integrin subunit obtained with vehicle cells (*black histogram*), RAR alpha agonist (*blue histogram*), RAR beta agonist (*red histogram*), RAR gamma agonist (*green histogram*), or an antibody isotype control (*gray histogram*) are shown. (B) The extent of mAb MB 1.2 (anti- $\beta 1$) binding to K562 cells exposed to vehicle, 1 μ M RAR α agonist, RAR β agonist, or RAR γ agonist for 24, 48, 72, or 96 hrs was assessed by FACS analysis. MB 1.2 (anti- $\beta 1$) mAb was used at a concentration of 10 μ g/mL. Cell surface levels of the $\beta 1$ integrin subunit obtained with vehicle cells (*black histogram*), RAR alpha agonist (*blue histogram*), RAR beta agonist (*red histogram*), RAR gamma agonist (*green filled histogram*), or an antibody isotype control (*gray histogram*) are shown.

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data demonstrates no differences in the $\alpha 5$ integrin subunit expression on the cell surface when K562 cells were exposed to either RAR α , RAR β , or RAR γ agonists for 24, 48, 72, or 96 hours (Fig 3A). However in contrast to the cell surface $\alpha 5$ integrin subunit findings, K562 cells exposed to the RAR γ agonist had an increase in cell surface $\beta 1$ integrin subunit expression compared to the vehicle within 48 hours (Fig 3B). Interestingly, K562 cell cultured with the RAR γ agonist demonstrate an increase in the cell surface $\beta 1$ integrin expression at 72 hours when compared to vehicle. At 96 hours, RAR γ agonist treated cells have an increase in cell surface $\beta 1$ integrin subunit levels compared to vehicle. Cell surface levels of the $\beta 1$ integrin subunit is comparable to the vehicle when K562 cells are exposed to the RAR α or RAR β agonists for 48, 72 or 96 hours. Our results shown in Fig 3B add support to the cellular adhesion assay findings shown in Fig 1A–1C. Collectively, our data demonstrate that the RAR γ agonist promotes cellular adhesion to $\alpha 5\beta 1$ integrin ligands through augmenting cell surface $\beta 1$ integrin expression.

RAR γ agonist exposure dampens K562 cellular proliferation in a time- and concentration-dependent manner

The balance between cellular adhesion and proliferation is fundamental to maintaining cell homeostasis with cells requiring anchorage to extracellular matrix proteins to proliferate. Interestingly, there have been studies demonstrating that unoccupied $\alpha 5\beta 1$ integrin causes cell growth inhibition [31, 32, 37]. Additionally, studies have demonstrated that over-expression of

the $\alpha 5\beta 1$ integrin results in suppressed cellular proliferation [29–32]. Due to the novel cellular adhesion response elicited by the RAR γ agonist and the corresponding increase in the integrin $\beta 1$ cell surface expression upon RAR γ agonist exposure, we assessed the impact of this agonist on K562 cellular proliferation. K562 cells were cultured in the presence of vehicle, RAR α agonist, RAR β agonist, or RAR γ agonist for 24, 48, 72, or 96 hours and proliferation levels were assessed as shown in Fig 4A. We observe a 18% decrease in proliferation levels when cells were treated with the RAR γ agonist, while cells cultured with either the RAR α or RAR β agonist had comparable proliferation levels to the vehicle at 24 hrs. At 48 hours, proliferation levels were decreased by 24% in the presence of the RAR γ agonist when compared to the vehicle. Interestingly, a similar trend was noted at 72 and 96 hours with a decrease in proliferation levels of 19% and 25%, respectively when K562 cells were treated with the RAR γ agonist. At 48, 72 and 96 hours, cells treated with either the RAR α or RAR β agonists had comparable proliferation levels to the vehicle.

It is well established that retinoids are modulators of proliferation. A number of studies have focused on the role of specific RARs in regulating proliferation with a limited number of investigations focusing on the RAR γ agonist at high concentrations [36, 38–40]. Due to the novel adhesion response demonstrated at lower concentrations of the RAR γ agonist, we assessed whether the same RAR γ agonist concentrations impact cellular proliferation. K562 cells were cultured in the presence of the RAR γ agonist for 72 hours at concentrations ranging from 125 nM–1 μ M. As shown in Fig 4B, we observe a slight, but significant decrease in proliferation compared to vehicle when cells were cultured with the RAR γ agonist at the lowest dose of 125 nM. A similar trend was observed when cells were treated with either the 250 nM or 500 nM RAR γ agonist with a decrease in cellular proliferation of approximately 10% compared to vehicle control. Interestingly, we observe a significant and large decrease in cellular proliferation when cells are dosed at the higher concentration of 1 μ M of the RAR γ agonist. Collectively, our data demonstrate a significant decrease in cellular proliferation when K562 cells are exposed to the RAR γ agonist that is time and concentration dependent.

Discussion

The requirement of vitamin A in maintaining cell homeostasis, particularly in regards to immunity has been appreciated since the earliest studies in the 1920's [41]. However, the molecular mechanisms by which retinoids elicit their effects was not illuminated until the discovery of retinoid receptors [11–16]. The current study focuses on the effects of RAR specific agonists on cellular adhesion and proliferation in the human erythroleukemia cell line, K562. We present evidence that the RAR γ agonist augments cellular adhesion to RGD containing extracellular matrix proteins fibronectin and FN-120, which contain the binding sites for $\alpha 5\beta 1$ integrins in a time- and concentration-dependent manner. Recently, K562 cells have been demonstrated to be retinoid response in the presence of troglitazone, a PPAR γ ligand [18]. In the presence of troglitazone, K562 cellular adhesion is dampened; however, upon the addition of the RAR/RXR pan-agonist, 9-*cis*-RA, cellular adhesion is restored to levels comparable vehicle treatment alone. Further, 9-*cis*-RA increases cell surface levels of the integrin subunit $\alpha 5$ when cells were exposed to troglitazone [18], while cell surface integrin subunit $\beta 1$ levels remain unchanged. Our current work may provide insight into how retinoids modulate both alpha 5 and beta 1 integrin subunit expression. Since integrins function as heterodimeric partners, we suggest that RAR γ retinoid receptor is responsible for the up-regulation of the beta 1 subunit, while the alpha 5 subunit may be transcriptionally regulated by the pan-agonist 9-*cis*-RA. It is plausible to suggest that through differential RAR/RXR pairings or promiscuous RXR partnerships different integrin subsets may be expressed to modulate cellular functions

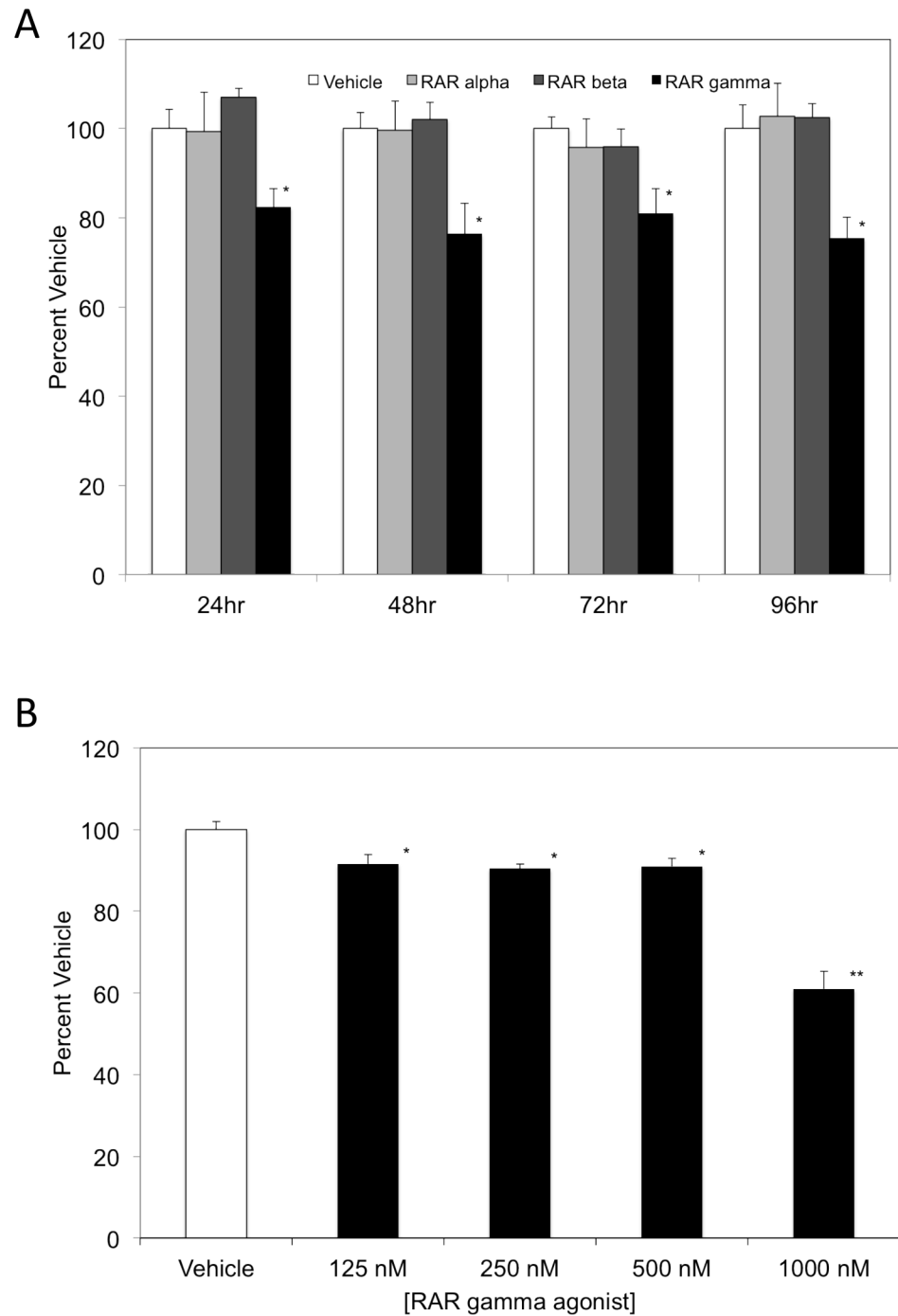


Fig 4. K562 cellular proliferation is decreased by RAR γ agonist exposure in a time- and concentration-dependent manner. (A) K562 cells cultured for 72 hrs in the presence of vehicle (white bars), 1 μ M RAR α agonist (light gray bars), RAR β agonist (dark gray bars) or RAR γ agonist (black bars), respectively, for 24, 48, 72 or 96 hrs. BrdU incorporation was assessed by ELISA and proliferation values were normalized using Percent Vehicle = (Abs_(treatment)/Abs_(vehicle)) X 100. Results are shown as means \pm SD, n = 12, LSD was used to test for differences among groups. Means followed by * are significantly different (P = 0.01). (B) K562 cells were cultured for 72 hrs in the presence of vehicle (white bars) or RAR γ agonist at varying concentrations (black bars). BrdU incorporation was assessed by ELISA and proliferation values were normalized using Percent Vehicle = (Abs_(treatment)/Abs_(vehicle)) X 100. Results are shown as means \pm SD, n = 6, LSD was used to test for differences among groups. Means followed by * or ** are significantly different (P = 0.01).

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including cellular adhesion and proliferation, which is required for proper cell homeostasis. Additionally, the RAR γ receptor isotype has been shown to differentially recruit specific components of the transcriptional machinery [42]. Both of these mechanisms may be involved in the augmented cellular adhesion observed in the presence of the RAR γ agonist.

All-*trans*-retinoic acid and 9-*cis*-retinoic acid have been implicated in modulating integrin-dependent and -independent cellular adhesion [18, 33, 35, 43]. Interestingly, we observe a slight but significant increase in cellular adhesion when cells were exposed to the RAR α agonist for 96 hours on the fibronectin ligand (Fig 1B). However, we do not observe an increase in cellular adhesion on the α 5 β 1 integrin specific FN-120 ligand in the presence of the RAR α agonist (Fig 1C). Further, we do not detect an observable change in the cellular surface levels of the alpha 5 or beta 1 integrin subunit expression (Fig 3A and 3B) in the presence of the RAR α agonist. Fibronectin contains binding sites for α 5 β 1, α v β 3 and α 4 β 7 integrins. Yet, K562 cells have not been reported to contain the α 4 β 7 integrin [35]. In a recent study, a number of integrins were profiled in K562 cells exposed to 9-*cis*-RA [18]. This study demonstrated that 9-*cis*-RA exposure did not increase α 8, β 3, α v β 5, or α v β 3 integrins on the cell surface of K562 cells and this data reflects very low surface levels regardless of vehicle or 9-*cis*-RA treatment group. Recently, the RAR α specific isotype has been shown to induce integrin-independent cellular adhesion in RPMI 8866 cells [43]. It is plausible to suggest K562 cells exposed to the RAR α agonist for 96 hours may have acquired integrin-independent adhesion strategies that are dependent on classical retinoid induced genetic modifications. Given the promiscuous nature of retinoid receptors and the complex signal transduction pathways involved in integrin-dependent and -independent cellular adhesion and migration, it is likely that distinct retinoid receptor pairings may facilitate a number of cellular events that modulate cellular adhesion and proliferation.

Our data reflect that the RAR γ isotype augments cellular adhesion and β 1 integrin cell surface expression, while dampening cellular proliferation. It is interesting to note that there appears to be a reciprocal relationship between cellular adhesion and proliferation that is mediated through the RAR γ isotype. Within the past three decades, the mechanisms by which retinoids elicit their classical genomic effects have been clearly established. There is a commonality between RAR isotypes as evidence by their ability to modulate transcription of common target genes. However, there is a disparity in retinoid receptor isotype functions [40]. For example, the RAR γ receptor isotype has been shown to differentially recruit specific components of the transcriptional machinery [42]. Additionally, there has been evidence of antagonism between RAR isotypes with RAR γ inhibiting functions of other RAR isotypes [40]. Moreover, different retinoid receptor isotypes have different transcriptional efficiency for the same target gene [40]. Further, others have demonstrated that low doses of selective RAR agonists acting in concert with their heterodimeric RXR partner may have more restricted effects on *t*-RA dependent transcription of target genes, thus avoiding the large number of adverse pleiotropic effects of *t*-RA [44]. Clearly, there is paucity of information regarding how RAR isotypes function to modulate the complex interplay between cellular adhesion and proliferation; however, determining the role that the RAR γ specific isotype plays in these integrin dependent processes warrants further investigation.

A precise balance between cellular adhesion and proliferation is required for cell homeostasis, particularly in the regulation of immunity. Retinoids are well established in their ability to regulate proliferation in a variety of cell types, and their role in modulating cellular adhesion is becoming more clear. Our results shown in Fig 4A are consistent with a previous study in K562 cells utilizing the RAR gamma agonist, CD437 [45]. Further, this same study reports that within 24 hours CD437 increases expression of p21, a cyclin-dependent kinase inhibitor. [45]. Interestingly, it has been demonstrated that overexpression of the beta 1 integrin subunit

results in a marked increase in the expression level of p21 [46]. A number of studies have suggested that p21 is capable of inducing apoptosis (for review see [47]). Hsu et al report that apoptosis was observed in K562 cells after 144 hours of exposure to CD 437 [45]. It is plausible to suggest that the RAR γ agonist, CD 437, may be increasing expression of both p21 and the beta 1 integrin and synergistically they may play a role in cellular growth arrest and potentially apoptosis at later exposure times.

The current study provides insight into the role of RAR specific isotypes in K562 cellular adhesion and proliferation. Our work is the first to demonstrate that treatment with the RAR γ specific agonist augments cellular adhesion in a time- and concentration- dependent manner to α 5 β 1 integrin substrates, increases cell surface levels of the β 1 integrin subunit, and dampens cellular proliferation in a time and concentration dependent manner in a human erythroleukemia cell line. To shed light on how this particular agonist may synergistically, yet reciprocally regulate cellular adhesion and proliferation, future studies will need to characterize the specific RAR γ -RXR partnerships involved in cellular adhesion and proliferation, determine how the α 5 β 1 integrin ligation status effects proliferation in the presence of this agonist, and identify the particular cellular signals responsible for the reciprocal regulation between cellular adhesion and proliferation that is propagated by retinoid treatment. The therapeutic use of retinoic acid is largely limited due to its numerous side effects; however, retinoids through their associated RARs play a pivotal role in controlling immunity. Illuminating the role of each RAR isotype would allow for strategic targeting of these receptors, which would profoundly impact immune regulation.

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Author Contributions

Conceptualization: MDK.

Data curation: MDK RP ML VN RM.

Formal analysis: MDK RP ML VN RM.

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References

1. Sporn MB RA, Goodman DS. *The Retinoids Biology, Chemistry, and Medicine*. New York: Raven Press; 1994.
2. Dillehay DL, Li W, Kalin J, Walia AS, Lamon EW. In vitro effects of retinoids on murine thymus-dependent and thymus-independent mitogenesis. *Cell Immunol*. 1987; 107(1):130–7. Epub 1987/06/01. PMID: [3495345](#).
3. Ertesvag A, Naderi S, Blomhoff HK. Regulation of B cell proliferation and differentiation by retinoic acid. *Semin Immunol*. 2009; 21(1):36–41. Epub 2008/08/16. S1044-5323(08)00056-0 [pii] <https://doi.org/10.1016/j.smim.2008.06.005> PMID: [18703353](#).
4. Cariati R, Zancai P, Quaia M, Cutrona G, Giannini F, Rizzo S, et al. Retinoic acid induces persistent, RARalpha-mediated anti-proliferative responses in Epstein-Barr virus-immortalized b lymphoblasts carrying an activated C-MYC oncogene but not in Burkitt's lymphoma cell lines. *International journal of cancer Journal international du cancer*. 2000; 86(3):375–84. PMID: [10760826](#).
5. Blomhoff HK. Vitamin A regulates proliferation and apoptosis of human T- and B-cells. *Biochemical Society transactions*. 2004; 32(Pt 6):982–4. Epub 2004/10/28. BST0320982 [pii] <https://doi.org/10.1042/BST0320982> PMID: [15506942](#).
6. Wu Y, Chen Q, Pai T, Ross AC. All-trans-retinoic acid and Erk1/2 signaling synergistically regulate the expression of CD300B in human monocytic cells. *Cell Immunol*. 2011; 268(2):68–78. Epub 2011/04/01. S0008-8749(11)00049-9 [pii] <https://doi.org/10.1016/j.cellimm.2011.03.002> PMID: [21450279](#);
7. Semba RD. The role of vitamin A and related retinoids in immune function. *Nutr Rev*. 1998; 56(1 Pt 2): S38–48. Epub 1998/03/03. PMID: [9481123](#).
8. Brown DC, Tsuji H, Larson RS. All-trans retinoic acid regulates adhesion mechanism and transmigration of the acute promyelocytic leukaemia cell line NB-4 under physiologic flow. *Br J Haematol*. 1999; 107(1):86–98. Epub 1999/10/16. PMID: [10520028](#).
9. Iwata M. The roles of retinoic acid in lymphocyte differentiation. *Semin Immunol*. 2009; 21(1):1–50. Epub 2008/10/08. <https://doi.org/10.1016/j.smim.2008.09.001> PMID: [18838279](#).
10. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity*. 2004; 21(4):527–38. Epub 2004/10/16. <https://doi.org/10.1016/j.immuni.2004.08.011> PMID: [15485630](#).
11. Brand N, Petkovich M, Krust A, Chambon P, de The H, Marchio A, et al. Identification of a second human retinoic acid receptor. *Nature*. 1988; 332(6167):850–3. Epub 1988/04/28. <https://doi.org/10.1038/332850a0> PMID: [2833708](#).
12. Giguere V, Ong ES, Segui P, Evans RM. Identification of a receptor for the morphogen retinoic acid. *Nature*. 1987; 330(6149):624–9. Epub 1987/12/17. <https://doi.org/10.1038/330624a0> PMID: [2825036](#).
13. Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, et al. 9-cis retinoic acid stereoisomer binds and activates the nuclear receptor RXR alpha. *Nature*. 1992; 355(6358):359–61. Epub 1992/01/23. <https://doi.org/10.1038/355359a0> PMID: [1309942](#).
14. Mangelsdorf DJ, Ong ES, Dyck JA, Evans RM. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature*. 1990; 345(6272):224–9. Epub 1990/05/17. <https://doi.org/10.1038/345224a0> PMID: [2159111](#).
15. Petkovich M, Brand NJ, Krust A, Chambon P. A human retinoic acid receptor which belongs to the family of nuclear receptors. *Nature*. 1987; 330(6147):444–50. Epub 1987/12/03. <https://doi.org/10.1038/330444a0> PMID: [2825025](#).
16. Chambon P. A decade of molecular biology of retinoic acid receptors. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*. 1996; 10(9):940–54. Epub 1996/07/01. PMID: [8801176](#).
17. Escribese MM, Conde E, Martin A, Saenz-Morales D, Sancho D, Perez de Lema G, et al. Therapeutic effect of all-trans-retinoic acid (at-RA) on an autoimmune nephritis experimental model: role of the VLA-4 integrin. *BMC Nephrol*. 2007; 8:3. Epub 2007/01/26. <https://doi.org/10.1186/1471-2369-8-3> PMID: [17250768](#);
18. Hanson AM, Gambill J, Phomakay V, Staten CT, Kelley MD. 9-cis-retinoic acid and troglitazone impacts cellular adhesion, proliferation, and integrin expression in K562 cells. *PLoS one*. 2014; 9(3):e93005. Epub 2014/03/29. <https://doi.org/10.1371/journal.pone.0093005> PMID: [24671180](#);
19. Massimi M, Devirgiliis LC. Adhesion to the extracellular matrix is positively regulated by retinoic acid in HepG2 cells. *Liver Int*. 2007; 27(1):128–36. Epub 2007/01/24. <https://doi.org/10.1111/j.1478-3231.2006.01391.x> PMID: [17241391](#).
20. Medhora MM. Retinoic acid upregulates beta(1)-integrin in vascular smooth muscle cells and alters adhesion to fibronectin. *Am J Physiol Heart Circ Physiol*. 2000; 279(1):H382–7. Epub 2000/07/19. PMID: [10899079](#).

21. Assoian RK, Schwartz MA. Coordinate signaling by integrins and receptor tyrosine kinases in the regulation of G1 phase cell-cycle progression. *Current opinion in genetics & development*. 2001; 11(1):48–53. PMID: [11163150](#).
22. Schwartz MA, Assoian RK. Integrins and cell proliferation: regulation of cyclin-dependent kinases via cytoplasmic signaling pathways. *Journal of cell science*. 2001; 114(Pt 14):2553–60. PMID: [11683383](#).
23. Pierschbacher MD, Ruoslahti E. Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule. *Nature*. 1984; 309(5963):30–3. Epub 1984/05/03. PMID: [6325925](#).
24. Garcia AJ, Vega MD, Boettiger D. Modulation of cell proliferation and differentiation through substrate-dependent changes in fibronectin conformation. *Mol Biol Cell*. 1999; 10(3):785–98. Epub 1999/03/09. PMID: [10069818](#);
25. Ingber D. Integrins as mechanochemical transducers. *Curr Opin Cell Biol*. 1991; 3(5):841–8. Epub 1991/10/01. PMID: [1931084](#).
26. Kinashi T. Overview of integrin signaling in the immune system. *Methods Mol Biol*. 2011; 757:261–78. Epub 2011/09/13. https://doi.org/10.1007/978-1-61779-166-6_17 PMID: [21909918](#).
27. Molla A, Block MR. Adherence of human erythroleukemia cells inhibits proliferation without inducing differentiation. *Cell Growth Differ*. 2000; 11(2):83–90. Epub 2000/03/14. PMID: [10714764](#).
28. Zhang Y, Wang H. Integrin signalling and function in immune cells. *Immunology*. 2012; 135(4):268–75. Epub 2012/01/04. <https://doi.org/10.1111/j.1365-2567.2011.03549.x> PMID: [22211918](#);
29. Dedhar S. Regulation of expression of the cell adhesion receptors, integrins, by recombinant human interleukin-1 beta in human osteosarcoma cells: inhibition of cell proliferation and stimulation of alkaline phosphatase activity. *Journal of cellular physiology*. 1989; 138(2):291–9. <https://doi.org/10.1002/jcp.1041380210> PMID: [2521862](#).
30. Dedhar S, Argraves WS, Suzuki S, Ruoslahti E, Pierschbacher MD. Human osteosarcoma cells resistant to detachment by an Arg-Gly-Asp-containing peptide overproduce the fibronectin receptor. *The Journal of cell biology*. 1987; 105(3):1175–82. PMID: [2443508](#);
31. Symington BE. Fibronectin receptor overexpression and loss of transformed phenotype in a stable variant of the K562 cell line. *Cell regulation*. 1990; 1(9):637–48. PMID: [2150333](#);
32. Varner JA, Emerson DA, Juliano RL. Integrin alpha 5 beta 1 expression negatively regulates cell growth: reversal by attachment to fibronectin. *Mol Biol Cell*. 1995; 6(6):725–40. Epub 1995/06/01. PMID: [7579691](#);
33. Bridges LC, Lingo JD, Grandon RA, Kelley MD. All-trans-retinoic acid induces integrin-independent B-cell adhesion to ADAM disintegrin domains. *Biochemistry*. 2008; 47(15):4544–51. Epub 2008/03/22. <https://doi.org/10.1021/bi702447u> PMID: [18355041](#).
34. Faull RJ, Kovach NL, Harlan JM, Ginsberg MH. Affinity modulation of integrin alpha 5 beta 1: regulation of the functional response by soluble fibronectin. *The Journal of cell biology*. 1993; 121(1):155–62. Epub 1993/04/01. PMID: [8458867](#);
35. Whelan JT, Chen J, Miller J, Morrow RL, Lingo JD, Merrell K, et al. 9-cis-retinoic acid promotes cell adhesion through integrin dependent and independent mechanisms across immune lineages. *J Nutr Biochem*. 2012; 24(5):832–41. Epub 2012/08/29. S0955-2863(12)00143-X [pii] <https://doi.org/10.1016/j.jnutbio.2012.05.001> PMID: [22925918](#);
36. Valli C, Paroni G, Di Francesco AM, Riccardi R, Tavecchio M, Erba E, et al. Atypical retinoids ST1926 and CD437 are S-phase-specific agents causing DNA double-strand breaks: significance for the cytotoxic and antiproliferative activity. *Molecular cancer therapeutics*. 2008; 7(9):2941–54. <https://doi.org/10.1158/1535-7163.MCT-08-0419> PMID: [18790775](#).
37. Giancotti FG, Ruoslahti E. Elevated levels of the alpha 5 beta 1 fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell*. 1990; 60(5):849–59. PMID: [2155708](#).
38. Guo Y, Lee YC, Brown C, Zhang W, Usherwood E, Noelle RJ. Dissecting the role of retinoic acid receptor isoforms in the CD8 response to infection. *Journal of immunology*. 2014; 192(7):3336–44. <https://doi.org/10.4049/jimmunol.1301949> PMID: [24610012](#);
39. Vivat-Hannah V, You D, Rizzo C, Daris JP, Lapointe P, Zusi FC, et al. Synergistic cytotoxicity exhibited by combination treatment of selective retinoid ligands with taxol (Paclitaxel). *Cancer research*. 2001; 61(24):8703–11. PMID: [11751388](#).
40. Bosch A, Bertran SP, Lu Y, Garcia A, Jones AM, Dawson MI, et al. Reversal by RARalpha agonist Am580 of c-Myc-induced imbalance in RARalpha/RARgamma expression during MMTV-Myc tumorigenesis. *Breast cancer research: BCR*. 2012; 14(4):R121. <https://doi.org/10.1186/bcr3247> PMID: [22920668](#);
41. Mellanby E, Green HN. Vitamin a as an Anti-Infective Agent: Its Use in the Treatment of Puerperal Septicaemia. *Br Med J*. 1929; 1(3569):984–6. Epub 1929/06/01. PMID: [20774721](#);

42. Gillespie RF, Gudas LJ. Retinoic acid receptor isotype specificity in F9 teratocarcinoma stem cells results from the differential recruitment of coregulators to retinoic response elements. *The Journal of biological chemistry*. 2007; 282(46):33421–34. <https://doi.org/10.1074/jbc.M704845200> PMID: [17875646](https://pubmed.ncbi.nlm.nih.gov/17875646/).
43. Whelan JT, Wang L, Chen J, Metts ME, Nasser TA, McGoldrick LJ, et al. Retinoids induce integrin-independent lymphocyte adhesion through RAR-alpha nuclear receptor activity. *Biochemical and biophysical research communications*. 2014; 454(4):537–42. <https://doi.org/10.1016/j.bbrc.2014.10.120> PMID: [25450689](https://pubmed.ncbi.nlm.nih.gov/25450689/).
44. Roy B, Taneja R, Chambon P. Synergistic activation of retinoic acid (RA)-responsive genes and induction of embryonal carcinoma cell differentiation by an RA receptor alpha (RAR alpha)-, RAR beta-, or RAR gamma-selective ligand in combination with a retinoid X receptor-specific ligand. *Molecular and cellular biology*. 1995; 15(12):6481–7. PMID: [8524212](https://pubmed.ncbi.nlm.nih.gov/8524212/);
45. Hsu CA, Rishi AK, Su-Li X, Gerald TM, Dawson MI, Schiffer C, et al. Retinoid induced apoptosis in leukemia cells through a retinoic acid nuclear receptor-independent pathway. *Blood*. 1997; 89(12):4470–9. PMID: [9192771](https://pubmed.ncbi.nlm.nih.gov/9192771/).
46. Fang Z, Fu Y, Liang Y, Li Z, Zhang W, Jin J, et al. Increased expression of integrin beta1 subunit enhances p21WAF1/Cip1 transcription through the Sp1 sites and p300-mediated histone acetylation in human hepatocellular carcinoma cells. *J Cell Biochem*. 2007; 101(3):654–64. <https://doi.org/10.1002/jcb.21223> PMID: [17211849](https://pubmed.ncbi.nlm.nih.gov/17211849/).
47. Gartel AL, Tyner AL. The role of the cyclin-dependent kinase inhibitor p21 in apoptosis. *Molecular cancer therapeutics*. 2002; 1(8):639–49. PMID: [12479224](https://pubmed.ncbi.nlm.nih.gov/12479224/).