Dexamethasone-Associated Cross-Linked Actin Network Formation in Human Trabecular Meshwork Cells Involves $\beta 3$ Integrin Signaling

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PURPOSE. To determine whether cross-linked actin networks (CLANs) formed in dexamethasone (DEX)-treated human trabecular meshwork (HTM) cells are structurally similar to those formed after $\beta 3$ integrin activation and involve $\alpha \nu \beta 3$ integrin signaling.

METHODS. Two HTM cell strains and an $\alpha \nu \beta 3$ integrin-overexpressing immortalized TM cell line were used. DEX- or ethanol-pretreated HTM cells were plated on fibronectin with or without $\beta 3$ integrin-activating mAb AP-5. Immunofluorescence microscopy was used to identify phalloidin-labeled CLANs and to ascertain the presence of α -actinin, PIP₂, and syndecan-4 within them. $\beta 3$ Integrin signaling involvement was determined using a PI3-kinase (LY294002) or Rac1 (NSC23766) inhibitor. $\alpha \nu \beta 3$ Integrin expression levels and the $\beta 3$ integrin activation state were determined by fluorescence-activated cell sorter analysis and immunofluorescence microscopy.

RESULTS. CLANs associated with either DEX treatment or $\beta 3$ integrin activation contained syndecan-4, PIP₂, and α -actinin. In the absence of mAb AP-5, LY294002 did not affect DEX-associated CLAN formation, whereas NSC23766 decreased the percentage of CLAN-positive cells by 80%. In the presence of mAb AP-5, both inhibitors decreased DEX-associated CLAN formation. DEX pretreatment increased $\beta 3$ integrin-induced CLAN formation nearly sixfold and the level of $\alpha \nu \beta 3$ integrin expression and activation threefold compared with control cells. Activated $\beta 3$ integrin-positive adhesions increased nearly fivefold in DEX-treated cells. $\alpha \nu \beta 3$ Integrin overexpression in TM-1 cells increased CLAN formation twofold.

CONCLUSIONS. DEX-associated CLANs were structurally similar to those induced by mAb AP-5 and involved both increased expression and activation of $\alpha \nu \beta 3$ integrins. Thus, glucocorticoid-induced CLAN formation may involve enhanced $\beta 3$ integrin signaling in HTM cells, possibly by an inside-out signaling mechanism. (*Invest Ophthalmol Vis Sci.* 2011;52:2952-2959) DOI:10.1167/iov.10-6618

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Actin-mediated processes play an important role in regulating aqueous humor outflow through the trabecular meshwork (TM).¹ In human and bovine TM cells, steroid treatment leads to increased formation of a novel actin structure called a cross-linked actin network (CLAN).^{2,3} This structure has been suggested to participate in the pathogenesis of steroid-induced glaucoma (SIG)^{2,4,5} and possibly primary open angle glaucoma.⁶ The exact role of CLANs in these diseases, however, has not been defined.

CLANs were originally observed in spreading cells and were described as actin geodesic domes.⁷ Although they have been found within the TM of healthy eyes, they appear to be more abundant in glaucomatous meshworks.^{8,9} More recently, CLANs have been observed in human and bovine lamina cribrosa cells that were either steroid treated or glaucomatous.¹⁰ CLANs are composed of interconnected arrays of three to five actin filaments extending outward from a central vertex. They may be precursors to actin stress fibers⁷ that regulate contractility in cells. Tropomyosin, α -actinin, and filamin were found along the actin filaments in CLANs, whereas the transmembrane heparan sulfate proteoglycan syndecan-4, phosphatidylinositol 4,5-bisphosphate (PIP₂), and α -actinin appear to form a molecular complex, or vertisome, at the vertices of the actin filaments.^{7,11-13} CLANs appear to be attached to the apical cell surface,^{11,12,14,15} presumably by syndecan-4. CLANs are often found within lamellipodia as cells spread, but they can also be found specifically over the nucleus or throughout the cytoplasm.^{2,7,15}

Treatment with glucocorticoids such as dexamethasone (DEX) increases CLAN formation in confluent monolayers of TM cells^{2,5,16} and in the TM of cultured eyes.¹⁷ CLAN formation can also be induced by activating a cooperative $\beta 1/\beta 3$ integrin signaling pathway that uses, in part, the G-protein-coupled receptor CD47 as a coreceptor coupled to the Rac1 GTPase and the guanine nucleotide exchange factor Trio.^{13,18}

Integrins belong to a ubiquitously expressed family of transmembrane heterodimeric glycoproteins composed of an α and a β subunit. The extracellular domain binds a number of extracellular matrix (ECM) proteins through the conserved sequence, arg-gly-asn, whereas their cytoplasmic tails interact with a variety of tyrosine kinases, adaptor proteins, and actin-binding proteins.^{19,20} As a result, integrins form an important physical link between the extracellular environment and the actin cytoskeleton and may provide a mechanism for sensing changes in external forces in the microenvironment of the TM.

In this study, we compared the roles of DEX and $\alpha \nu \beta 3$ integrins in CLAN formation. These studies show that CLANs induced by DEX treatment are structurally similar to those formed by $\beta 3$ integrins and that CLAN formation involves the same signaling pathway activated by $\alpha \nu \beta 3$ integrins. We also show that DEX treatment increases the level of expression and activation of $\beta 3$ integrin and that overexpression of $\alpha \nu \beta 3$

integrin can increase CLAN formation. These studies suggest that glucocorticoids may trigger CLAN formation by enhancing $\beta 3$ integrin signaling in HTM cells through the upregulation of $\beta 3$ integrins or the activation of an inside-out signaling mechanism that triggers $\alpha v \beta 3$ signaling. Elucidating the signaling pathways that direct the formation and disassembly of CLANs in the TM may further our understanding of SIG and other glaucomas.

MATERIALS AND METHODS

Materials

The monoclonal antibody (mAb) syndecan-4 (150.9) was kindly provided by Anne Woods (University of Alabama, Birmingham, AL). mAb AT6.172 (α -actinin), mAb G-A-5 (glial fibrillary-acidic protein [GFAP]), rabbit anti-F-actin, and dexamethasone were purchased from Sigma (St. Louis, MO). mAb KT10 against PIP₂ was purchased from Assay Designs, Inc. (Ann Arbor, MI). mAb M9 (αv integrin), mAb PM6/13 ($\beta 3$ integrin), mAb LM609 ($\alpha v \beta 3$ integrin), and purified mouse IgG₁ were purchased from Millipore Corp. (Temecula, CA). mAb CRC54 (activated $\beta 3$ integrin) was purchased from Abcam Inc. (Cambridge, MA). Alexa 488-conjugated phalloidin, Alexa 488-conjugated goat anti-rabbit IgG, Alexa 488-conjugated goat anti-mouse IgG, Alexa 546-conjugated goat anti-mouse IgG, and Hoechst 33342 were all purchased from Invitrogen (Carlsbad, CA). mAb AP-5 against $\beta 3$ integrin was purchased from The Blood Center of Wisconsin (Milwaukee, WI). The PI-3 kinase inhibitor LY294002 and the Rac1 inhibitor NSC23766 were both purchased from EMD Chemicals (Gibbstown, NJ). The pcDNA3 expression vector was purchased from Invitrogen. The pcDNA3- αv and pcDNA3- $\beta 3$ vectors were kindly provided by Mark H. Ginsberg (University of California, San Diego, CA).

Cell Culture

The N27TM-1 and N27TM-2 strains of HTM cells were isolated from two 27-year-old donors as previously described^{21–23} and were cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma), 15% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 2 mM L-glutamine (Sigma), 1% amphotericin B (Mediatech, Herndon, VA), 0.05% gentamicin (Mediatech), and 1 ng/mL FGF-2 (PeproTech, Rocky Hill, NJ). Three to 7 days after reaching confluence, HTM monolayers were treated with 500 nM DEX or vehicle control (0.1% ethanol) for 4 days in media lacking FGF-2 before they were used in spreading assays. In some experiments, subconfluent cultures of proliferating cells were treated with ethanol only or DEX for 4 to 7 days before they became confluent, at which time they were used in spreading assays. The immortalized TM cell line TM-1²⁴ was routinely cultured in low-glucose DMEM, 10% FBS, 2% L-glutamine, 1% amphotericin B, and 0.05% gentamicin without FGF-2.

Spreading Assays

Confluent HTM monolayers pretreated with either DEX or vehicle were serum-starved for 24 hours and then replated in the continued presence of either vehicle or 500 nM DEX onto coverslips precoated with 20 nM fibronectin, as previously described,^{13,18} in the presence of 25 μ g/mL cycloheximide. Individual experiments were performed with duplicate determinations except where noted. In some experiments, cells were preincubated with 20 μ M LY294002 or 60 μ M NSC23766 for 1 hour before replating on fibronectin-coated coverslips. Ten minutes before replating, 8 μ g/mL of the $\beta 3$ integrin-activating mAb AP-5 was added to the cells. Cells were allowed to spread for 1.5 to 2 hours (N27TM-1 and N27TM-2) or 3 hours (TM-1) before fixation in either 4% p-formaldehyde alone for 10 minutes, 4% p-formaldehyde + 0.2% Triton X-100 for 30 minutes, or -20°C methanol for 15 minutes, depending on the immunolabeling to be performed. Cells that were fixed in 4% p-formaldehyde alone were treated after fixation with 0.2% TX-100 in PBS for 5 minutes.

Immunofluorescence Microscopy and Quantification of CLANs and Integrin-Positive Cells

Fixed cells were labeled with either Alexa 488-conjugated phalloidin together with antibodies against activated $\beta 3$ integrins or $\alpha v \beta 3$ integrins (paraformaldehyde-fixed cells) or polyclonal anti-F-actin together with antibodies against GFAP (as a negative control) α -actinin, syndecan-4, or PIP₂ (methanol-fixed cells). Anti-F-actin antibodies were detected using Alexa 488-conjugated goat anti-rabbit IgG, whereas all monoclonal antibodies were detected using Alexa 546-conjugated goat anti-mouse IgG. All cells were labeled with Hoechst 33342 to localize nuclei. Fluorescence was observed with an epifluorescence microscope (Axioplan 2; Zeiss, Thornwood, NY) equipped with a digital camera (AxioCam HRm; Zeiss) and image acquisition software (Axiovision version 4.6; Zeiss).

To quantify CLAN-positive cells (CPCs), five to seven low-power (200 $\times$) fluorescence images from each coverslip were captured. The minimum requirement for an actin structure to be counted as a CLAN was as previously described.¹⁸ Data were pooled from two to four

FIGURE 1. Effects of DEX treatment on CLAN formation in HTM cells treated with or without the $\beta 3$ integrin-activating antibody AP-5. Quiescent (A) or proliferating (B) HTM cultures were treated with 0.1% ethanol (EtOH) or 500 nM DEX for 4 days (A) or 4 to 7 days (B). Cells were plated and spread on fibronectin $\pm$ mAb AP-5 for 1.5 to 2 hours before fixation and labeling with phalloidin. The percentage of CPCs was determined and was represented as the mean $\pm$ SD. (A) *n* ranged from 3502 to 3544 cells. (B) *n* ranged from 2015 to 3732 cells. (A, B) Data were pooled from experiments performed with two HTM cell strains. The increases in the DEX only-treated cells relative to control cells at 4 (A) and 4 to 5 (B) days were statistically significant at $P < 0.05$. All other changes were statistically significant at $P < 0.01$.

experiments and represented the mean percentage of CPCs $\pm$ the SD of the mean. To quantify integrin-positive cells (IPCs), six images from each coverslip were captured, and the number of cells containing integrin-positive adhesion complexes was counted along with the total number of cells to calculate the percentage of IPCs per image. Statistical analysis comparing the different treatment groups for CLAN formation or adhesion complex formation was performed using ANOVA. Where pairs of treatment groups had to be compared, ANOVA analysis was used in conjunction with the Tukey HSD test.

Fluorescence-Activated Cell Sorter Analysis

Fluorescence-activated cell sorter analysis (FACS) was performed on TM-1 and HTM cells, as previously described.²⁵ Cells were lifted from tissue culture dishes nonenzymatically using a commercial product (Cell Dissociation Solution; Sigma) or 2 mM EDTA before they were blocked with TBS/1% goat serum. Cells were then incubated with antibodies against α v, β 3, or α v β 3 integrins or a purified mouse IgG1 control prepared to 10 μ g/mL in 1% BSA/Tris-buffered saline (TBS) for 30 minutes, on ice. Cells were then incubated with Alexa 488-conjugated goat anti-mouse IgG at 5 μ g/mL in 1% BSA/TBS for 30 minutes, on ice. Analysis was performed with a flow cytometer (FACSCalibur System; BD Biosciences, San Jose, CA). The geometric means of each peak were normalized by subtraction of the mean of the appropriate IgG control peak. Changes in integrin levels in response to DEX treatment were assessed by comparing the normalized geometric means of the vehicle and DEX treatment peaks.

Transfection of TM-1 Cell Line

TM-1 cells were plated in six-well plates and grown to 70% confluence. Cells were transfected with 2.5 μ g pcDNA3- α v or pcDNA3- β 3 using a plasmid DNA delivery system (Lipofectamine LTX with PLUS Reagent; Invitrogen) according to the manufacturer's protocol. In some experiments, cells were cotransfected with 1.25 μ g pcDNA3- α v and 1.25 μ g

pcDNA3- β 3. Twenty-four hours after transfection, cells were switched to serum-free medium. After another 24-hour incubation, cells were either used in a spreading assay to measure CLAN formation or subjected to FACS analysis to determine cell surface expression of integrins. This experiment was performed three times using triplicate determinations.

RESULTS

Analysis of Dexamethasone-Associated CLAN Formation

Previous studies indicated that CLANs formed by the activation of an integrin-dependent signaling pathway consisted of interconnecting arrays of actin filaments radiating outward from central vertices that contained α -actinin, syndecan-4 and PIP₂.^{7,13} To determine whether CLANs formed in DEX-treated HTM cells were structurally similar, HTM cells pretreated with DEX or the ethanol vehicle were replated in the presence or absence of the β 3 integrin-activating antibody AP-5. Figure 1A shows that pretreatment of confluent, quiescent cells for 4 days with DEX increased ($P < 0.05$) CLAN formation from 1% in ethanol-treated control cells to 5%. The same increase ($P < 0.05$) was observed in subconfluent, proliferating cells treated for up to 5 days with DEX compared with control cells (Fig. 1B). Longer DEX treatments increased ($P < 0.01$) CLAN formation to 10% of cells, whereas the percentage of CLANs in ethanol-treated cells remained unchanged (Fig. 1B).

To determine whether these CLANs were structurally similar to those induced by β 1/ β 3 integrin signaling in the absence of DEX,¹³ cells treated with DEX for 7 days before spreading on fibronectin were double-labeled for F-actin and either α -actinin, syndecan-4, or PIP₂. As shown in Figure 2, α -actinin,

FIGURE 2. Colocalization of F-actin and α -actinin, PIP₂, or syndecan-4 in CLANs formed in the presence of DEX. HTM cells treated with 500 nM DEX for 7 days were plated and spread on fibronectin before fixation. The cells were double-labeled with antibodies against F-actin (A–D) and GFAP (a negative control) (A), α -actinin (B), PIP₂ (C), or syndecan-4 (D). Arrows: points of colocalization of F-actin and α -actinin, PIP₂, or syndecan-4 in CLAN vertices.¹³ Scale bar, 20 μ m.

syndecan-4, and PIP₂ were localized in the vertices of CLANs, indicating that DEX-associated CLANs are structurally similar to those induced by the activation of $\beta 1/\beta 3$ integrin signaling in the absence of DEX.¹³ Cells pretreated with vehicle only for 7 days and spread on fibronectin in the presence of mAb AP-5 demonstrated identical CLAN-labeling patterns for α -actinin, syndecan-4, and PIP₂, respectively, as those described in Figure 2 (data not shown).

Effects of Dexamethasone on CLAN Formation Induced by the Activation of $\alpha v \beta 3$ Integrins

Although DEX alone induced a small but significant increase in CLAN formation above vehicle-treated cells, DEX pretreatment increased the level of CLANs formed by activating $\alpha v \beta 3$ integrins to a much greater extent. In ethanol-treated cells, 20% ($P < 0.01$) of the cells formed CLANs in the presence of the $\beta 3$ integrin-activating mAb AP-5 (Fig. 1A), while pretreating cells with DEX increased the mAb AP-5-induced response to 30% ($P < 0.01$). In the absence of mAb AP-5, 1% of the ethanol-treated cells formed CLANs.

The effect of DEX was not limited to quiescent cells because DEX also enhanced the ability of $\alpha v \beta 3$ integrins to induce CLAN formation in proliferating cells (Fig. 1B). In proliferating cells treated for 4 to 7 days with vehicle only, the percentage of cells forming CLANs increased from <1% in ethanol-treated controls to ~8% ($P < 0.01$) in the presence of the mAb AP-5. This increase in CLAN formation was similar to that observed in cells treated with DEX alone in the absence of the AP-5 antibody. Depending on the length of pretreatment, DEX increased ($P < 0.01$) CLAN formation induced by mAb AP-5 in proliferating cells to 28% or 44% of cells, respectively. Thus, pretreatment with DEX significantly enhanced the ability of $\alpha v \beta 3$ integrins to trigger CLAN formation in both quiescent and proliferating HTM cells. This suggests that DEX pretreatment was activating some component of the $\alpha v \beta 3$ integrin signaling pathway used to form CLANs.

We also assessed the effects of treatment with DEX or mAb AP-5, or both, on the formation of multiple CLANs within single cells. There was no discernible difference in the number of CLANs per cell versus the treatment. In all the treatment groups (mAb AP-5 only, DEX only, or DEX + mAb AP-5), >60% of the CLAN-positive cells contained only one CLAN, whereas the other CLAN-positive cells had no more than two CLANs per cell (data not shown).

Role of $\alpha v \beta 3$ Integrin and Rac1 Signaling in Dexamethasone-Associated CLAN Formation

To determine whether CLANs formed by DEX treatment involved integrin signaling, DEX-treated HTM cells were incubated with the Rac1 inhibitor NSC23766 or the PI3-kinase inhibitor LY294002. Previous studies showed that integrin-dependent CLAN formation involved the convergence of distinct $\beta 1$ and $\beta 3$ integrin signaling pathways.¹⁸ The $\beta 1$ integrin pathway was PI-3 kinase dependent, whereas the $\beta 3$ integrin pathway involved the Rac1-specific guanine nucleotide exchange factor Trio. In the absence of the $\beta 3$ integrin-activating mAb AP-5, the Rac1 inhibitor NSC23766, which blocks the activation of Rac1 by Trio, caused a statistically significant ($P < 0.01$) decrease in the percentage of CLAN-positive cells in DEX-treated cells from 4% to <1% (Fig. 3A). In contrast, the PI3-kinase inhibitor LY294002 had no effect. Similarly, NSC23766 significantly ($P < 0.01$) reduced CLAN formation induced by mAb AP-5 in the absence or presence of DEX (Fig. 3B). The NSC23766 inhibitor reduced CLAN formation by 50% to 60%, respectively, which suggests that DEX pretreatment was primarily affecting CLAN formation activated by $\beta 3$ integrin signaling. LY294002 had no effect on $\beta 3$ integrin-mediated

FIGURE 3. CLAN formation in DEX-treated HTM cells is dependent on Rac1/Trio signaling. HTM cells were treated with 0.1% ethanol or 500 nM DEX for 4 days and then plated and spread on fibronectin for 1.5 to 2 hours in the absence (A) or presence (B) of mAb AP-5 $\pm$ the PI-3 kinase inhibitor LY294002 or the Rac1/Trio inhibitor NSC23766. Cells were fixed and labeled with phalloidin, and the percentage of CPC was determined. Results are represented as the mean $\pm$ SD. (A) The increase in the DEX only-treated cells relative to control cells and the decrease due to treatment with the Rac1 inhibitor relative to its control were statistically significant at $P < 0.01$. There were no statistically significant differences between the other treatment groups. n ranged from 1649 to 1863 cells. (B) The decrease in AP-5-induced CLAN formation after treatment with the NSC23766 inhibitor was statistically significant at $P < 0.01$ in both DEX-treated and ethanol-treated cells. In the presence of DEX, the decrease in AP-5-induced CLAN formation with the LY294002 inhibitor was statistically significant at $P < 0.01$ relative to the DMSO-treated control. In the presence of ethanol only, the decrease in AP-5-induced CLAN formation after treatment with the LY294002 inhibitor was statistically significant at $P < 0.05$ relative to the untreated control. There was no difference between cells treated with ethanol and AP-5 relative to ethanol, AP-5, and DMSO or between cells treated with ethanol, AP-5, and DMSO relative to those treated with ethanol, AP-5, and LY294002. n ranged from 1441 to 1576 cells.

CLAN formation induced by mAb AP-5 in ethanol-treated cells, which is consistent with our earlier studies.¹⁸ However, the LY294002 inhibitor did decrease $\beta 3$ integrin-mediated CLAN formation in DEX-treated HTM cells induced by mAb AP-5 compared with both the control untreated cells ($P < 0.05$) and the DMSO control-treated cells ($P < 0.01$).

$\alpha v \beta 3$ Integrin Expression/Activation in Dexamethasone-Associated CLAN Formation

Gene microarray studies²⁶ have suggested that $\beta 3$ integrin expression in HTM cells is upregulated by DEX. To determine whether DEX affected integrin expression, we examined the level of $\alpha v \beta 3$ expression in DEX-treated cells using the mAbs LM609 and CRC54, respectively. The monoclonal antibody LM609 recognizes all $\alpha v \beta 3$ integrins,²⁷ whereas mAb CRC54

FIGURE 4. Increased adhesion complex localization of $\alpha v\beta 3$ and activated $\beta 3$ integrin in DEX-treated HTM cells. (A) N27TM-2 HTM cells treated with 0.1% ethanol (A1, A2, A5, A6) or 500 nM DEX (A3, A4, A7, A8) for 4 days were plated and spread on fibronectin for 1.5 to 2 hours before fixation. The cells were double-labeled with mAb LM609 (A1, A3) and phalloidin (A2, A4) to localize $\alpha v\beta 3$ integrins and actin, respectively, or with mAb CRC54 (A5, A7) and phalloidin (A6, A8) to localize activated $\beta 3$ integrins and actin, respectively. Scale bar, 20 μ m.

FIGURE 5. FACS analysis of HTM cells for DEX-induced changes in the expression and activation state of $\alpha v\beta 3$ integrins. HTM cells were treated with ethanol (–DEX) or 500 nM DEX for 4 days before FACS analysis. Cells were lifted nonenzymatically and were labeled with nonspecific mouse IgG (solid gray peaks) or with either anti- $\alpha v\beta 3$ or anti-activated $\beta 3$ integrins (open peaks). Primary antibodies were detected with Alexa 488-conjugated anti-mouse IgG.

specifically recognizes the activated state of $\beta 3$ integrins.²⁸ Immunofluorescence studies show that, in the absence of the $\beta 3$ integrin activating mAb AP-5, DEX treatment significantly increases the percentage of cells containing both $\alpha v\beta 3$ integrin and activated $\beta 3$ integrin in adhesion complexes compared with treatment with ethanol alone (Fig. 4). $\alpha v\beta 3$ Integrin-positive cells increased from 20.5% to 64% ($P < 0.01$), and activated $\beta 3$ integrin-positive cells increased from 12% to 56% ($P < 0.01$) after DEX treatment. This suggests that DEX drives the localization of activated $\alpha v\beta 3$ integrins into adhesion complexes. FACS analysis verified that both the levels of total $\alpha v\beta 3$ integrin expression and the level of activated $\beta 3$ integrin increased after treatment with DEX. Figure 5 shows that activated $\beta 3$ integrin levels were increased threefold by DEX and that total $\alpha v\beta 3$ integrin levels increased fourfold.

To determine whether the overexpression of $\alpha v\beta 3$ could increase CLAN formation, TM-1 cells,²⁴ which express negligible levels of $\alpha v\beta 3$ integrin and respond poorly to mAb AP-5 with regard to CLAN formation (Figs. 6A, 6B), were cotransfected with pcDNA3- αv and pcDNA3- $\beta 3$ expression plasmids. FACS analysis indicated that cotransfection of both integrin subunits was needed to increase surface expression of $\alpha v\beta 3$ integrin (data not shown). As shown in Figure 6A, cotransfection of αv and $\beta 3$ integrin expression plasmids resulted in elevated levels of $\alpha v\beta 3$ integrins and CLAN formation. CLAN formation increased from 5% in control cells to 9% in αv - and $\beta 3$ -cotransfected TM-1 cells in the absence of mAb AP-5 ($P < 0.05$) and from 7% to 16% in the presence of mAb AP-5 ($P < 0.05$).

(B) The percentage of ethanol- or DEX-treated HTM cells with activated $\beta 3$ -integrins (open bars) or total $\alpha v\beta 3$ -integrins (black bars) in adhesions was determined and was represented as the mean $\pm$ SD. n ranged from 411 to 683 cells. DEX increased the percentage of activated $\beta 3$ IPCs and $\alpha v\beta 3$ IPC over their corresponding EtOH-treated control groups ($P < 0.01$). Data were pooled from experiments performed with both N27TM-1 and N27TM-2 HTM cells.

0.01). In contrast, CLAN formation did not significantly change in TM-1 cells transfected with only αv integrin or $\beta 3$ integrin compared with control cells, regardless of whether AP-5 was present (Fig. 6B). Together these data suggest DEX induction of CLAN formation may be attributed to the activation of the $\alpha v \beta 3$ signaling pathway previously shown to induce CLAN formation in HTM cells.^{13,18}

DISCUSSION

In this study, we show that DEX can activate the $\alpha v \beta 3$ integrin signaling pathway previously shown to trigger CLAN formation in HTM cells.^{13,18} DEX treatment led to increased levels of activated $\alpha v \beta 3$ integrins²⁹ that were clustered into adhesion complexes. Activation of this pathway by DEX also involved the Rac1/Trio signaling pathway previously shown to be associated with $\alpha v \beta 3$ -induced CLAN formation.¹⁸ The CLANs formed by either DEX treatment or direct activation of $\alpha v \beta 3$ integrins¹³ contained the same components, suggesting that they were structurally comparable. Given that CLANs have been proposed to alter the microarchitecture of the TM and subsequent contractile properties of the TM in glaucomatous eyes (both POAG and SIG),¹⁷ this would suggest that $\alpha v \beta 3$ integrin signaling may be involved in some of the changes observed in these glaucomas.

Structurally and morphologically, CLANs formed by DEX-mediated activation of integrins appeared to be similar to those previously observed in steroid-treated confluent monolayers.² Although very little is known about the molecular events that trigger CLAN formation in confluent cultures or in vivo, our present studies suggest that fibronectin-binding integrins are likely to be involved. Integrins, especially those that bind

fibronectin, are found on the TM cell surface in vivo and in DEX-treated confluent cultures. Fibronectin is also a major component of the matrix under both these conditions.^{24,30,31} Thus, fibronectin-binding integrins are likely to be engaged and to influence CLAN formation, especially in DEX-treated confluent monolayers in which fibronectin synthesis is upregulated.

Although $\alpha v \beta 3$ integrin signaling appears to be the primary pathway affected by DEX in this study, the possibility that it is also affecting $\beta 1$ integrin signaling cannot be completely ruled out. Previous studies showed that $\beta 1$ and $\beta 3$ integrins cooperate to induce CLAN formation in HTM cells through the use of distinct integrin signaling pathways.^{13,18} The $\beta 1$ pathway is sensitive to LY294002 and, thus, dependent on PI3-kinase, whereas the $\beta 3$ pathway is sensitive to NSC23766, indicating that it is dependent on Rac1/Trio.¹⁸ Because the PI-3 kinase inhibitor did not have any effect on CLAN formation in the presence of DEX alone, this suggested that $\beta 1$ integrin signaling did not contribute to the increased CLAN formation observed in the presence of DEX. In addition, NSC23766 decreased CLAN formation associated with DEX treatment down to the same level observed in control cells, suggesting that the primary signaling pathway involved $\alpha v \beta 3$ integrins. However, LY294002 did decrease CLAN formation at least 25% compared with controls when cells were treated with DEX and the $\beta 3$ integrin activating mAb AP-5. This suggested that although DEX by itself may not use $\beta 1$ integrins to form CLANs, the enhanced $\alpha v \beta 3$ integrin signaling caused by pretreatment with DEX may activate some additional $\beta 1$ integrin signaling, possibly by crosstalk^{32,33} between the two integrins.

We speculate that DEX activates $\alpha v \beta 3$ integrin signaling through an inside-out signaling mechanism (Fig. 7^{13,18,34}). Inside-out signaling³⁵ is regulated by intracellular signals, which

FIGURE 6. CLAN formation increases in TM-1 cells transfected with $\alpha v \beta 3$ integrin. (A) Immortalized TM-1 cells were transfected with αv or $\beta 3$ integrins, or both and lifted nonenzymatically with 2 mM EDTA in TBS before they were labeled with nonspecific mouse IgG, anti- αv , anti- $\beta 3$, or anti- $\alpha v \beta 3$. Primary antibodies were labeled with Alexa 488-conjugated goat anti-mouse and detected by FACS. (B) Transfected cells were plated and spread on fibronectin $\pm$ mAb AP-5 for 3 hours before fixation. Cells were labeled with phalloidin, and the CPC percentage was determined. The percentage of CPCs is represented as the mean $\pm$ SD; n ranged from 916 to 1452 cells. The increase in CLAN formation in $\alpha v \beta 3$ -cotransfected cells was greater than vehicle-treated, αv integrin-transfected, or $\beta 3$ integrin-transfected cells ($P < 0.01$), regardless of AP-5 treatment. In the presence of AP-5, the CPC percentage in cotransfected cells was greater than in cells not treated with AP-5 ($P < 0.01$).

FIGURE 7. Schematic diagram showing potential points at which DEX may affect β_3 integrin-mediated CLAN formation. Previous studies^{13,18} showed that CLAN formation could be induced by an outside-in signaling mechanism involving the AP-5 mAb, which directly activates β_3 integrin or the thrombospondin 1 (TSP-1) peptide 4N1K, which binds CD47. In this study, we showed that DEX may also influence integrin signaling by upregulating α_v or β_3 (or both) expression at the cell surface or by inducing the active state of $\alpha_v\beta_3$. Activation of $\alpha_v\beta_3$ could occur through regulatory molecule(s), such as talin,³⁴ that directly bind the cytoplasmic tails of β_3 integrins. Alternatively, DEX could upregulate the downstream effectors of β_3 integrin signaling such as Src or Rac1/Trio, previously shown to regulate β_3 -mediated CLAN formation.¹⁸ These latter two possibilities are considered to influence integrin signaling by an inside-out signaling mechanism.

induce conformational changes in the cytoplasmic tails of integrins that enhance the affinity of the integrin for its ligand. Consistent with this idea is the observation that, compared with control cells, DEX-treated HTM cells exhibited increased levels of activated β_3 integrins at the cell surface that were found within adhesion complexes. In addition, activation of the $\alpha_v\beta_3$ integrins appeared to be maintained in the absence of an extracellular ligand given that FACS analysis, performed with cells in suspension in the absence of any extracellular ligands, showed that cells exhibited a threefold increase in the level of activated $\alpha_v\beta_3$ integrins compared with ethanol-treated controls. This suggests that DEX triggers an intracellular signaling mechanism that induces the active conformation of $\alpha_v\beta_3$ integrin, possibly by promoting the interaction of proteins with the cytoplasmic tails of $\alpha_v\beta_3$ integrins that, in turn, initiate subsequent signaling cascades downstream of the integrin. Additionally, spreading assays were performed with cells pretreated with trypsin, which removed extracellular ligands bound to integrins and cycloheximide to inhibit de novo protein synthesis. Collectively, these studies support the idea that DEX treatment activates an intracellular signaling pathway that results in $\alpha_v\beta_3$ integrin activation and subsequent signaling.

Previous studies did not find that activating $\alpha_v\beta_3$ integrin with mAb AP-5 enhanced CLAN formation in DEX-treated cells.¹³ Yet, we observed a nearly sixfold enhancement of CLAN formation in some of the studies reported here. One possible explanation for this inconsistency is the timing of the assay. In this study we looked at CLAN formation in normal HTM cells at 1.5 to 2 hours rather than 3 hours,^{13,18} which suggests, as we have reported (Filla MS, et al. *IOVS* 2004;45: ARVO E-Abstract 4415), that there may be a time-dependent factor in CLAN formation in the presence of DEX. Alternatively, the difference could be due to cell strain specificity. Clark et al.² reported that there is variability between cell strains in their CLAN-forming ability after DEX treatment, which is consistent with the observation that not everyone is a steroid

responder.³⁶ Thus, it is possible that the cell strain used in the earlier study did not respond in the same manner as the two strains used in this study.

In summary, these data demonstrate that DEX can regulate integrin signaling in HTM cells and can provide some molecular insights into how the microarchitecture of the TM cytoskeleton and its functions may be altered in some glaucomas. Understanding the molecular pathways altered by DEX should help in the development of therapeutic treatments for steroid-induced glaucoma.

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