RESEARCH ARTICLE

Effect of Adenosine Agonists on the Proliferation and Differentiation of Chick Embryo Fibroblasts in Three Dimensional Reconstituted Tissue Constructs

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ABSTRACT

Previous studies indicate that organ fibroblasts play an important role in wound healing, collagen production, remodeling processes and pathogenesis of progressive heart, lung, renal and hepatic fibrotic diseases. Several studies suggest a possible inhibitory role for adenosine in the regulation of fibroblast proliferation. The effect of adenosine A_2 agonists on proliferation and differentiation of chick embryo skin/muscle fibroblasts was studied in collagen-based 3-dimensional tissue constructs and also in plated monolayer cells. Materials and Methods: Chick embryo primary fibroblasts were plated in separate groups and were synchronized by growth arrest before stimulation by different doses of adenosine, and A_2 receptor agonists, CV1808, NECA and an A_2 receptor antagonist, CGS15943, and control, in the presence of serum or serum free medium. The cell counts for each treatment of monolayer fibroblasts were compared to determine fibroblast proliferation. Western blot analysis, immunostaining and myofibroblast size measurements were conducted to measure the effect of adenosine on the fibroblast differentiation into myofibroblasts. Cell proliferation was also gauged with DNA assays in the 3-D constructs. Results: Adenosine agonists at low doses significantly reduced fibroblast proliferation in monolayer and 3-D cell culture in the presence of 5% Fetal Calf Serum (FCS) demonstrating a potential antifibrotic activity possibly by activation of the A_{2B} receptor. Western blot analysis and immunostaining of cells revealed no significant inhibition of the expression of α-smooth muscle actin on a per cell basis by adenosine agonists. Cell size measurements indicated increased numbers of smaller fibroblasts suggesting that adenosine may inhibit the conversion of fibroblasts to myofibroblasts. Conclusion: This study suggests that agents that increase tissue cAMP levels may be of beneficial therapeutic value in organ tissue fibrosis.

Keywords: *Chick embryo fibroblasts, Proliferation, Differentiation, Adenosine agonists*

Myofibroblasts play a central role in the formation of fibrotic tissue. These cells are related to fibroblasts and exhibit a hybrid phenotype between fibroblasts and smooth muscle cells. They are characterized by expression of α-Smooth Muscle Actin (α-SMA) and increased collagen production. Following an injury, the myofibroblasts play a major role in wound closure and the process of wound healing. After wound repair, myofibroblasts are removed by selective apoptosis. Their abnormal persistence, is believed to be responsible for the excessive collagen production that leads to alteration of the tissue architecture and ultimately to organ failure [1].

Organ fibroblast growth is regulated by several autocrine/paracrine factors including adenosine which has long been known as a retaliatory metabolite particularly in the heart, where it induces cardioprotective effects [2]. The biological effects of adenosine are mediated by adenosine receptors, which exist in multiple subtypes (A_1, A_{2A}, A_{2B}, and A_3 receptors). Previous studies using A_{2B} receptor ($A_{2B}R$) antagonists as well as overexpression and antisense-mediated reduction of $A_{2B}R$ show that this receptor is responsible for the antimitogenic and antiproliferative effects of adenosine [3, 4].

The signalling mechanism responsible for A_{2B} R-mediated inhibition of protein synthesis and collagen release in cardiac fibroblasts (CF) is not fully known. $A_{2B}R$ is known to couple to Gs proteins and when activated can upregulate cAMP production. Increases in cAMP levels have been associated with the inhibition of cellular functions such as proliferation, DNA, protein synthesis and collagen release [5].
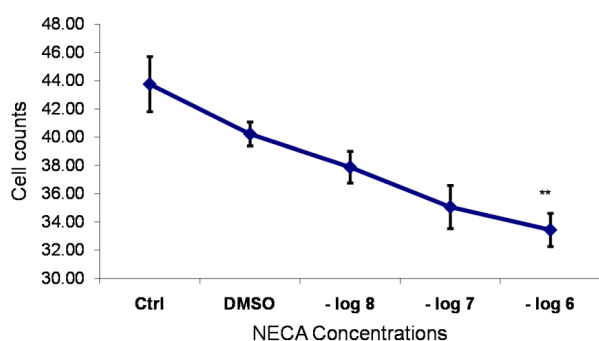
Fig 1. NECA at the doses of 10^{-8}–10^{-6} M presented an inhibitory effect on the 5% serum -induced proliferation of primary first passage CEF cells in a dose-dependent fashion.

Table 1. Comparison of the average cell Sizes in three groups of CEF cells of different treatments. The number of fibroblasts that were smaller than expected for myofibroblasts was greater in the adenosine/NECA-treated plates than control. Cells with large sizes (over 2 times) (representative of myofibroblasts) were significantly increased in the plates treated with the control or DMSO.

Type Of Cells/Treatment	L Cells %*	I Cells %**	S Cells %***	Total%
Control	23	54	12.5	100
DMSO	23	56	21	100
Adenosine	6	47	46	100
NECA	7	59	33	100

* large cells (31±3 μm)
** Intermediate Cells (18±5 μm)
*** Small cells (10±4 μm)

Utilizing molecular approaches there is evidence that human A_3R may be critically involved in the inhibition of proliferation [3].

$A_{2B}R$ couples to G proteins and when activated can upregulate cAMP production. However, as documented in other cell types, $A_{2B}R$ activation can also increase intracellular levels of Ca^{++} [6-8] that can lead to activation of other signaling pathways. Thus the effects of the $A_{2B}R$ may be mediated by the simultanous activation of different signal transduction pathways. There is also the possibility that $A_{2B}R$ activation may inhibit protein and/or collagen release through indirect actions such as the suppression of cytokine production [9].

Cyclic AMP, a ubiquitous second messenger produced in response to activation of adenylyl cyclase (AC), influences growth, death and differentiated functions of many cell types. These effects are primarily mediated by promoting protein phosphorylation via cAMP dependent protein kinase A (PKA). Previous studies have demonstrated a cross-talk between the mitogen-activated protein kinase (MAPK) pathway and the cAMP signaling pathway [10]. It has been shown that cAMP attenuates tyrosine kinase receptor-stimulated MAPK in cells such as smooth muscle cells treated with Platelet Derived Growth Factor homodimer (PDGF-BB) [11]. Fibroblasts are stimulated by EGF (Epidermal Growth Factor), LPA (Lysophosphatidic Acid) [12] [13], PDGF, or insulin [14] and adipocytes by insulin [15]. Inhibition of these responses appears to be mediated by PKA. G protein coupled receptor (GPCR) agonists that signal through Gs to activate AC and stimulate cAMP production can also inhibit collagen synthesis [2, 16, 17].

CTGF (Connective Tissue Growth Factor), a protein associated with fibrosis that is upregulated in response to TGF-β1 stimulation in kidney fibroblasts, is decreased by cAMP [18].

The precise mechanism by which AC activation inhibits fibroblast differentiation requires further study. Activation of the low molecular weight G protein Rho A plays a key role in proper formation and assembly of the actin cytoskeleton [19-21]. Rho A stimulates formin-induced assembly of actin [16], and cAMP, through the activation of PKA, can inactivate RhoA and promote loss of stress fibers [17, 22, 23].

It is possible that other effects are involved, such as blockade of α-SMA production at the level of gene expression: a transforming growth factor beta TGF-beta response element in the alpha- SMA promoter is required for alpha- SMA gene expression [23-25]. Recent data indicates that cAMP acts in a PKA dependent manner to inhibit TGF-β /Smad signaling and gene activation by disruption of transcriptional cofactor binding [19].

The purpose of this study, was to determine if cAMP, could modulate the proliferation of CEF cells and their transition to myofibroblasts as assessed by cell counting and α-SMA protein expression. In addition we examined the receptor mechanisms and studied the effect of agonists in different media and serum concentrations to ascertain how adenosine affects the cell number in different conditions . The proliferation assays were performed on both monolayer and 3-D collagen-based
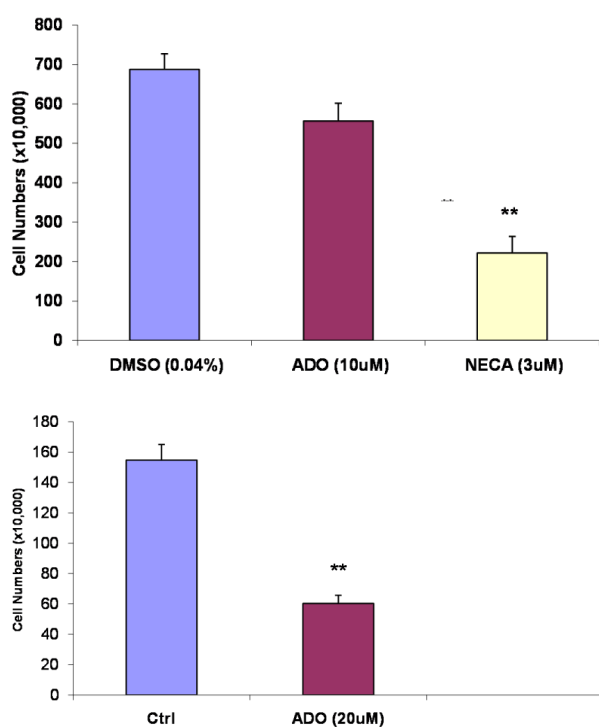

Fig 2. NECA and adenosine at the doses of 3×10^{-6}M and 2×10^{-5}M, respectively, produced a significant reduction in cell numbers following a 4- day administration to CEF cells in 5% serum.
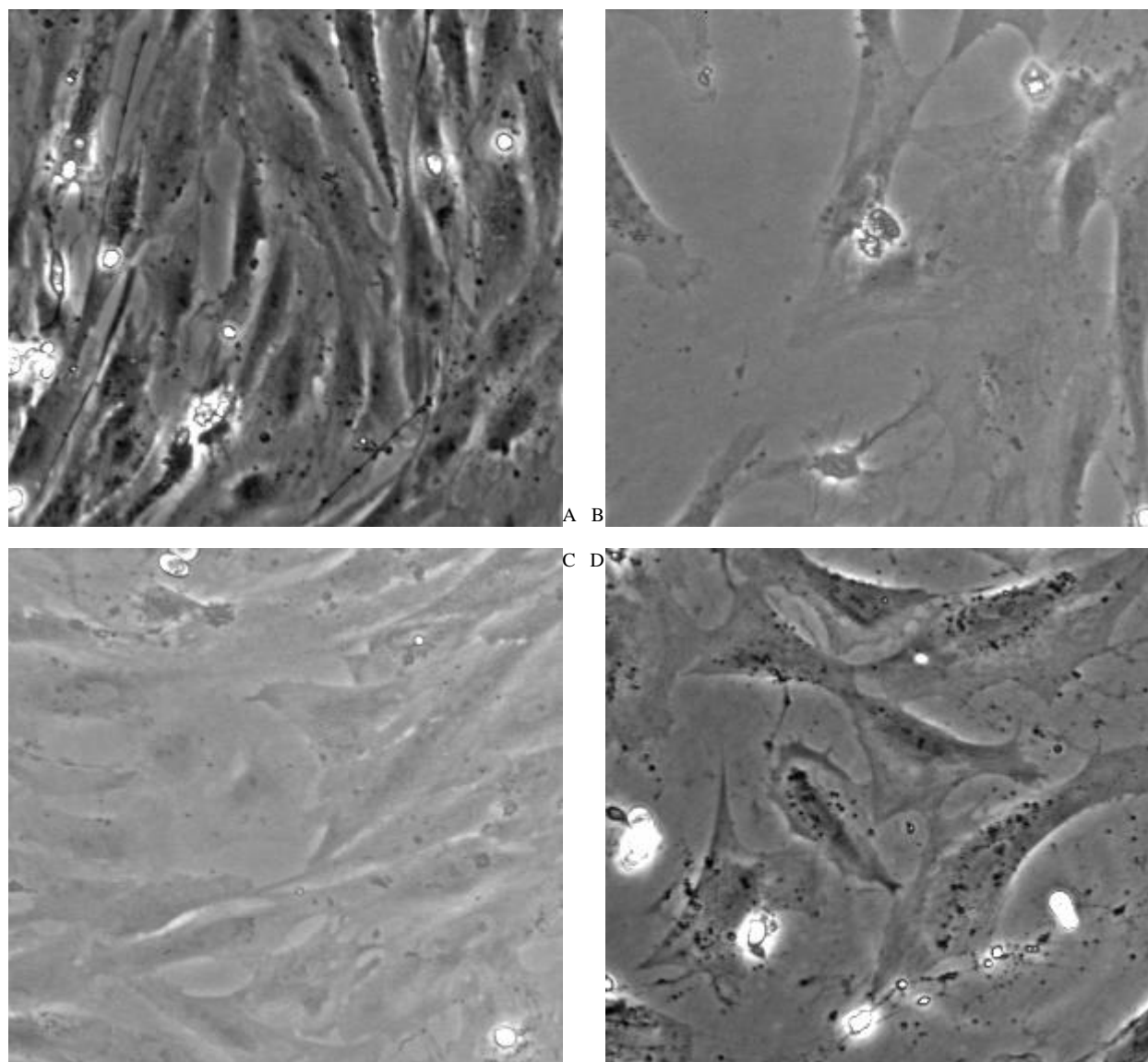
Fig 3. Micrograph of the fibroblasts under different treatments: confluent fibroblasts before stimulation (left-up) A, less confluent fibroblasts after five days stimulation with NECA (right-up) B, fibroblasts after 5 days stimulation with NECA+CGS 15 (left- down) C, fibroblasts stimulated with vehicle alone (DMSO 0.04M) (right-down) D, after 5-days treatment.

cell cultures. We utilized molecular/cellular approaches to ascertain a possible preventive effect of cAMP-producing agents in fibroconnective tissue diseases.

MATERIALS AND METHODS

Proliferation Assays

Chick Embryo Fibroblasts (CEFs) were obtained from ten-day-old eggs. After careful removal of the organs from the embryo, muscle and skin were dissected, minced, trypsinized, filtered and centrifuged. Pellets were suspended and the first passage or second passage of the CEFs, were plated at a density of 5×10^5 cells /plate in growth medium comprising Dulbeco's Modified Eagle Medium (DMEM) supplemented with 10% FCS. After 48 hours of incubation, cells were rendered quiescent in serum-free medium (SFM) for 48 hours before addition of medium containing 5% FCS supplemented with the adenosine agonists NECA [5'-(N-ethylcarboxamido) adenosine], adenosine, CV1808, an

adenosine antagonist, 9-chloro-2 (furanyl)- 5,6-dihydro-1,2,4-triazolo [1,5-c] quinazoline-5-imine (CGS 15943), dimethyl sulfoxide (DMSO) or control. Medium and drugs were replaced every day and cell number was determined after 4 days in triplicate using a hemocytometer. The cell count was in the blind plate assay system. Cell lysates from each treatment group were frozen for western blot analysis. Cells of each treatment group were fixed and stained for immunofluorescence microscopy using a Zeiss LSM-5 confocal microscope.

3-D Collagen Based Tissue Culture

First passage CEFs were cultured in 100×20 mm tissue culture dishes with DMEM supplemented with 5% FCS. The fibroblasts were then detached by 0.25% trypsin/EDTA solution and resuspended in DMEM solution. Collagen gels were prepared by mixing the appropriate amount of rat collagen type 1, FCS, DMEM and cell suspension [28]. The cell numbers and final volume of the collagen gels were 6×10^5 cells in 2 mL (1 mg/mL
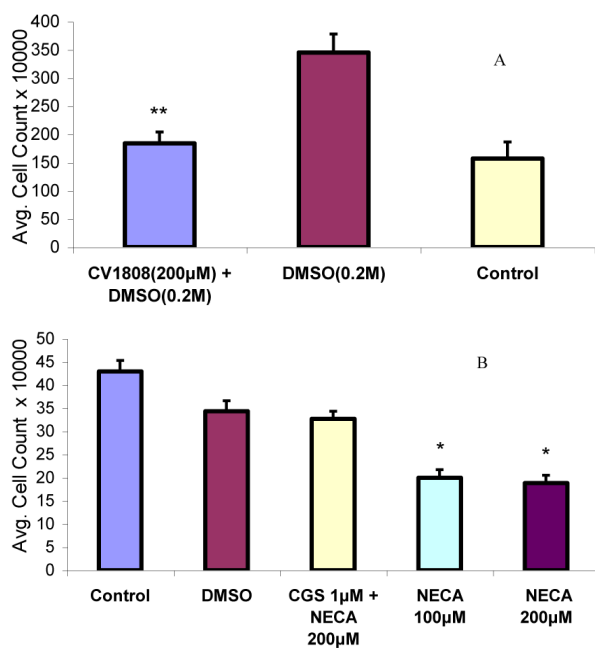
Fig 4. (A) Both adenosine agonists NECA and CV1808 at higher doses (100-200 µM) presented a significant reduction in numbers of CEF cells following a 4-day treatment in serum-free medium. (B) This effect was blocked by CGS 15943.

Fig 6. First and second passage CEF cells did not show a significant reduction in cell numbers following the administration of low-dose adenosine agonists in low serum (B) or serum-free medium (A). This figure presents even a protective effect of adenosine and its agonist NECA on the proliferation of CEF cells rather than a preventive effect.

collagen concentration). To form "button gels", 0.25 mL of the collagen- cell suspension was gently placed in the center of each well of a 6-well plate to form a hemispherical shape and incubated for 1 hour at 37°C. After the gelation was completed, a volume of fresh culture medium with 5% serum was placed on top of each gel. The medium was changed every other day. 3-D culture dishes were incubated for 24 hours before stimulation with NECA, adenosine, DMSO and control. Each treatment group was tested in triplicate and the experiment was repeated 3 times. After 8 days of daily administration of the agonists the gels were prepared for DNA assay.

Total DNA Quantification Assay for Collagen Gels

Each gel was transferred to an individual tube, washed with PBS and centrifuged. 1 mL of lysis buffer
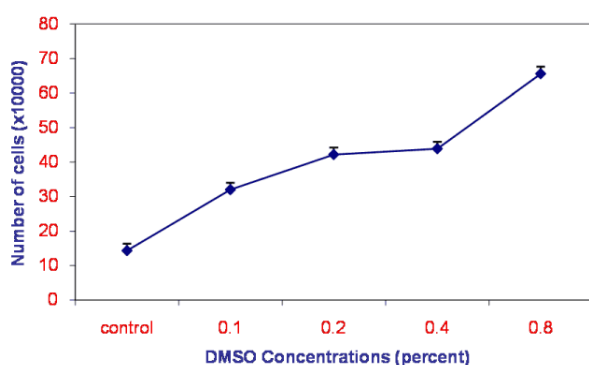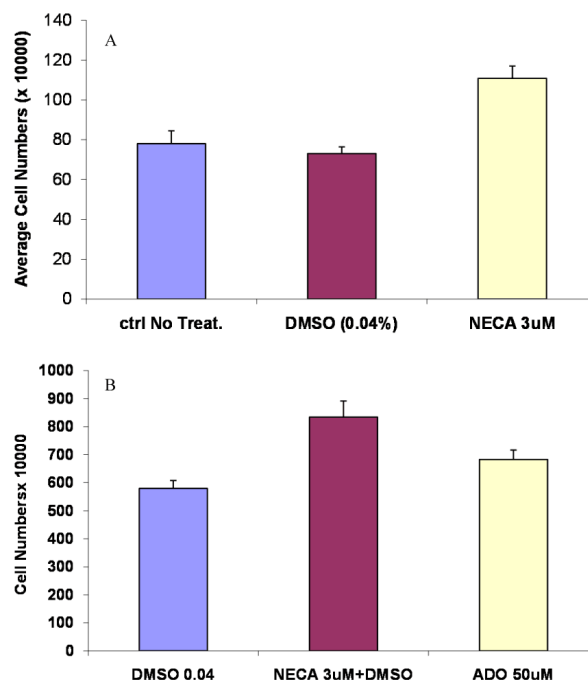
was added to each tube before sonicating (Branson B-3, 117 volts) for 10 minutes. DNA was assayed fluorimetrically with Hoechst dye 33258 (Sigma) [20].

Western Blotting

Samples were denatured with SDS loading buffer in boiling water for 10 minutes and then separated under reducing conditions on a polyacrylamide gel with a 5% stacking gel in SDS/Tris/glycine running buffer. The protein was electrophoretically transferred to a millipore membrane which was then blocked with 4% (w/v) non-fat milk in TTBS buffer (50 mmol/L Tris-HCl, and 1% v/v Tween 20) for 1 hour at room temperature under agitation. The membrane was then incubated in primary antibody at the suggested dilution (usually between 1:500–1:1000) in blocking buffer, overnight on a shaker at 4°C. The blot was then incubated with the secondary antibody at the suggested dilution (usually between 1:1000–1:5000 dil) supplemented with 4% nonfat milk for 1 hour with rocking, at room temp. The α-SMA was then detected using an enhanced chemiluminescence's detection system (Amersham) and exposed to x-ray film.

Rhodamine-Phalloidin Staining

The cells were rinsed in 4% paraformaldehyde in PBS (freshly prepared) for 15 minutes at room temperature (RT) and then washed in PBS for 5 minutes. The cells were permeabilized in 0.1% Triton-X100 and in PBS for 1 min. After rinsing with PBS the cells were blocked for 1 hour at RT. Primary antibody was applied (1/500) in 4% Bovine Serum Albumin BSA/PBS at RT
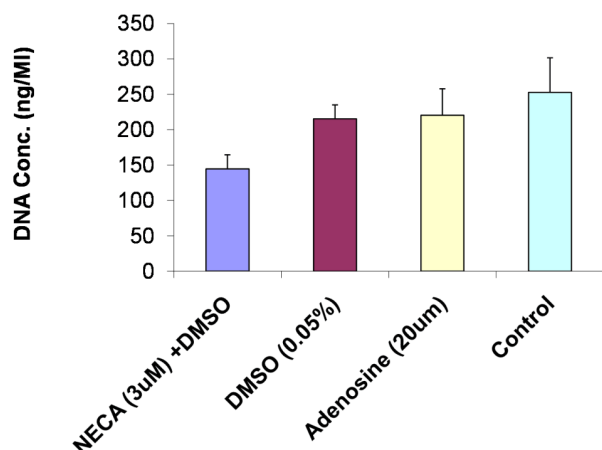
Fig 5. Dose-response effect of DMSO (vehicle for NECA and CV1808 on the proliferation of CEF cells. These doses of DMSO alone cause an increased proliferation of CEF cells.

Fig 7. Collagen-based 3-D CEF cell culture showed a mild inhibition of proliferation measured by a DNA assay following the tion of NECA and adenosine for 7 days. The proliferation of CEF cells was slower in the collagen-based tissue than the monolayer cells which may allow a delay in proliferation inhibition produced by adenosine and NECA in the 3-D cell culture.

for 1 hour. The sample was rinsed with PBS prior to application of the secondary antibody (FITC, TRITC-Conjugated) (1/2000) in 4% BSA/PBS at RT for 1 hour and the sample was prepared for confocal fluorescence microscopy.

Cell Size Measurements

Myofibroblasts are significantly larger than fibroblasts [21]. Micrographs were taken of the CEF cells in collagen gels and in monolayers exposed to each treatment group and cells sizes were measured using the following method:

After incubation for 7 days, micrographs were taken from the button gels (3-D collagen-based tissue constructs made from a collagen gel mixture of each treatment group [28]). Spherical cells exposed to each treatment observed in micrographs of monolayer cells on the hemocytometer were divided into three size categories according to their diameters, large (25-33 μm), intermediate (14-25 μm) and small cells (6-14 μm) as measured by a ruler. The number of cells in each size category in each treatment group was counted in the same surface area (Table 1). The differences in the numbers in each size category were compared. Statistical significance was measured by the student t-test at the level of $p<0.05$.

RESULTS

1. NECA at the doses of 10^{-8}–10^{-6} M inhibited (15.3%-20%) the serum -induced proliferation of primary second passage CEF cells in a dose - dependent fashion (Fig 1). NECA and adenosine at the doses of 10^{-6} and 2×10^{-5} produced a significant reduction in cell numbers following a 4-day exposure in 5% serum (Fig 2). NECA at the dose of 10^{-5} M did not show a significant effect on cell numbers.

2. Pre-administration of adenosine antagonist CGS 15943 prevented the effect of NECA and adenosine

in serum-induced proliferation of CEF cells (Fig 4B).

3. Both adenosine agonists NECA and CV1808 at higher doses (100-200 μM) caused a significant reduction in numbers of CEF cells following a 4- day treatment in serum- free medium (Fig 3 and 4). This effect was also blocked by CGS 15943 (Fig 3A and B). The total concentration of DMSO as a solvent for CV1808 was 0.4% to make a 100 μM NECA solution. This concentration of DMSO showed a significant increase in the cell number after a 4 day period of treatment. Therefore a dose-response curve for different concentrations of DMSO was obtained (Fig 5). DMSO showed a dose -dependent increase in cell numbers using 0.1%– 0.8% concentrations. Addition of CV1808 significantly decreased the CEF proliferation in serum -fee medium.

4. Second and third passage cells at the lower cell density showed similar responses to NECA, CV1808 and adenosine.

5. First and second passage CEF cells did not show a significant reduction in cell numbers following the administration of low-dose adenosine agonists in low or serum-free medium (Fig 6). In fact NECA and adenosine presents a protective effect on the cell population rather than a proliferation inhibition.

6. Collagen-based 3-D CEF cell culture showed a mild but not significant inhibition of proliferation measured by a DNA assay following the administration of NECA and adenosine for 7 days (Fig 7).

7. To determine the effect of each treatment on the stress fiber production, western blot analysis for the α-SMA concentration was carried out in 2-ways. First, equal volumes of the cell suspension of each treatment group (Control, DMSO, and NECA) were analyzed to confirm for the results of the cell proliferation assay. This showed a significant reduction of α-SMA in the NECA treated cells compared to the control. Second, westerns carried out in equal numbers of cells from each treatment group showed no change in α-SMA on a per/cell basis (Fig 8).

8. Measurements of the cell sizes for each treatment group showed a significant decrease in the sizes of cells following the treatment with adenosine and NECA (Table 1).

9. The amount and organization of total actin stained by rhodamine- phalloidin and of α- SMA (stained by the α-SMA antibodies) were similar for all treatment groups. (Images 1-4, available as supplementary material at web site).

10. Collectively, our findings indicate that adenosine agonists produce a significant reduction in the number of CEF cells in 3-D and monolayer cell culture in the presence of 5% serum, suggesting their potential for suppressing myofibroblast production. However, low serum (1.5%) or serum -free medium did not show a reduced cell number in the presence of adenosine agonists at the same doses, since cells do not show a growth and proliferation in low serum or serum free medium.
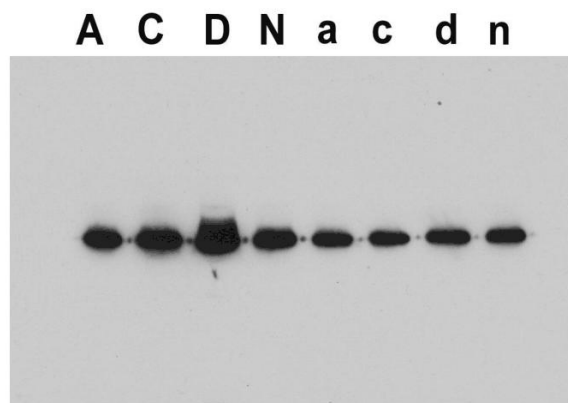
Fig 8. Western blot for α-smooth muscle actin demonstrating a difference between NECA treated cells (N) vs. DMSO (D) only when the sample taken was equal in volume. (A) Adenosine; (C) Control; (D) DMSO; (N) NECA. The samples don't show any difference for α-smooth muscle actin on a per cell basis on CEF cells under following treatments: a (adenosine), c (control), d (DMSO), n (NECA).

DISCUSSION

Our experiments showed that adenosine agonists produce a decrease in the number of chick embryo fibroblasts and/or myofibroblasts. NECA (adenosine A_1/A_{2B} R agonist) produced its maximum inhibitory effect on the serum-induced proliferation of CEF cells at the concentration of 2.5×10^{-6} M. The EC_{50} of NECA, one of the most potent $A_{2B}R$ agonists known, is 2×10^{-6} M. To show the involvement of the A2BR we used NECA at the dose range of 10^{-6}–10^{-4} M. The results from the concentrations between 10^{-8}–10^{-6} are contrasted to those obtained with 10-5 mol/L concentrations of NECA, (the concentrations at which A_1R is stimulated) and suggests that $A_{2B}R$ may play a prominent role in inhibiting cell proliferation in CEF cells [3].

The effect of NECA and adenosine on the proliferation of CEF cells in collagen- based cell culture showed a mild inhibition, as CEF proliferation in collagen gels is slow.

The inhibitory effect of high doses of agonists on the proliferation of fibroblasts in serum- free medium occurs as a toxic effect. The biochemical basis of adenosine toxicity has been studied in a number of cell lines. Extracellular adenosine has been reported to induce alterations in intracellular ATP and ADP levels which subsequently inhibit either orotate phosphoribosyl transferase, or the accumulation of the substrate for the enzyme phosphoribosyl pyrophosphate, thereby inhibiting de novo UTP and DNA synthesis [22].

NECA at the dose 2×10^{-6} M did not show a significant effect on the proliferation of CEF cells in low serum (1.5% FBS) and serum free conditions. In addition, trypan blue exclusion tests indicated no loss in viability, compared to control, indicating a regulatory role at this dosage for the compound in serum containing medium [5, 10].

$A_{2B}R$-mediated inhibition of proliferation and collagen synthesis not only mediates a stimulation of adenyl cyclase, but also couples to a PLC-dependent calcium signal [23].

According to the experiments conducted by Panjehpour group to show the involvement of intracellular Ca^{++} on the proliferation inhibition, the following data have been found: Activation of the phospholipase C pathway to produce cAMP and an intracellular Ca^{++} increase also may be responsible for proliferation inhibition. It has been shown that expression of $A_{2B}R$ caused a transient Ca^{++} signal in MDA-MB-231 cells. A signal of almost the same magnitude was observed in the absence of extracellular Ca^{++} although the signal was transient. The NECA response was antagonized in a concentration dependent manner by the A_1/A_{2B} antagonist DPCPX and the non-selective antagonist ANR 152. The subtype selective antagonists SCH 58261 (A_{2A}) and MRS1220 (A_3) had no effect on the NECA- induced Ca^{++} signal [23]. Calcium antagonists have been shown to inhibit proliferation. Thus, activation of IP3 pathway to produce more cAMP could be an alternative mechanism for A2BR action. On the other hand, if Ca^{++} signals are important in $A_{2B}R$-mediated inhibition of proliferation and differentiation, then Ca^{++} antagonists could prevent its antiproliferative action, whereas Ca^{++} antagonists have been shown to be proliferation inhibitors in some fibroblasts [24, 25].

Activation of adenyl cyclase activity through increased Ca^{++} levels attenuates cell proliferation and collagen synthesis [25]. Also it has been shown that the transcription enhancer factor is negatively regulated by Ca^{++} and cAMP [26]. Hence, the involvement of Ca^{++} in inhibitory function of the A2B receptor on the CEF proliferation remains to be further elucidated [3].

Adenosine agonists have been shown to have a preventive effect on the differentiation of fibroblasts, they are believed to have an inhibitory action on the production of α-SMA or stress fibers in fibroblasts. In our experiments western blot analysis did not reveal any per cell reduction of α-SMA in CEF cells. Previous studies have shown that fibroblasts rapidly differentiate into myofibroblasts (as indicated by increased α-SMA expression) when cultured on rigid substrates and/or in culture media containing serum and when plated at low density [27]. It has been found that untreated cardiac fibroblasts (CFs) spontaneously undergo this differentiation under normal culture conditions as shown by enhanced α-SMA expression in CF between passage 2 and passage 5. Thus adult rat CFs convert to myofibroblasts during early passage when plated on plastic tissue culture dishes in serum containing media emphasizing the importance of culture conditions in studies of the effects of exogenous agents on myofibroblast formation. For this reason, all differentiation studies should be conducted under serum- free conditions using low passage CFs (passage ≤ 2) that were plated at relatively high density (≈ 200 cells/mm2). In our experiments differentiation still occurred in the presence of NECA, adenosine or control. Thus different treatment groups did not show a significant difference regarding fibroblast differentiation. The present results may be influ-

enced by the effect of cell culture conditions on the conversion to myofibroblasts [27].

These results on the effect of cAMP on cell proliferation and differentiation suggest that cAMP producing agents may be beneficial for treatment of fibrosis in fibro-connective tissue diseases. The ability of adenosine agonists and cAMP producing agents such as adenosine agonists, PDE inhibitors, prostaglandins, and antidepressants to regulate differentiation of and collagen production by myofibroblasts represents a potentially important therapeutic value for the control of adverse tissue remodeling and fibrosis. Inhibition of cytokine-induced fibroblast activation, of the Rho kinase pathway, of PKA, and stimulation of the IP3 pathway by $A_{2B}R$ activation could reduce tissue distortion due to fibrosis and is considered as a key therapeutic goal in fibro- connective tissue disorders.

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