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Abstract

Hypothesis: We hypothesize that increase in activation of cyclic AMP (cAMP) levels will reduce both basal and PDGF-induced cell migration of Dupuytren's contracture-derived fibroblasts.

Method: Fibroblasts harvested from actively diseased Dupuytren's contracture cord (DC) and from the adjacent grossly unaffected palmar fascia in the same patients (PF) were compared to fibroblasts derived from the palmar fascia of CT patients. Cells treated with or without PDGF (2ng/ml) and/or forskolin (10 μM, a known cAMP inducer) were then subjected to an *in-vitro* wound healing "scratch" assay to measure cell motility into the denuded zone; photographs were taken at 0h and 48h and quantified.

Results: We found higher basal motility in DC- compared to unaffected palmar fascia (PF-) and CT-derived fibroblasts. PDGF stimulated cell motility in all three populations, the addition of forskolin inhibited both basal and PDGF-induced cell migration in all three cell types. Interestingly, the inhibitory effect of forskolin on PDGF-induced cell migration was more pronounced in DC-derived fibroblasts compared to the other two cell types. Western blot analysis showed that neither PDGF nor forskolin exposure had any effect on phosphorylation of p38 and PI3 kinase in DC-derived fibroblasts. Both forskolin and PDGF increased p42/44 MAP kinase phosphorylation. In contrast, elevated cAMP resulted in increased RhoA phosphorylation and a subsequent decrease in the level of activated RhoA in DC-derived fibroblasts.

Conclusions: We show that unaffected palmar fascia in DC is host to cells that are intermediate in phenotype between CT control and actively diseased DC cells. These results for the first time examine DC fibroblast motility and suggest that cAMP may be a useful agent to modulate DC fibroblast behavior and possibly forestall disease progression and recurrence. Take together these results imply that cAMP may be a useful agent to modulate DC fibroblast behavior and possibly forestall disease progression and recurrence.

Objectives

- The main objective of this study was to investigate the basal and PDGF-induced cell migration in CT-, PF- and DC-derived fibroblasts
- Our next objective was to determine if increase in growth factor-induced cell migration can be reduced by elevating cAMP levels.
- We also investigated the signaling pathways utilized by these cell lines during cell motility.

Introduction



Figure 1: Images of patients with Dupuytren's disease. Image courtesy: www.eatonhand.com/img/. Much more robust treatment strategy is required to reduce the recurrence rate of the disease.

Materials and Methods

Cell Cultures

Primary cultures of fibroblasts were obtained from the surgically resected DC, from matching specimens of normal appearing palmar fascia in Dupuytren's patients (PF), and from specimens of normal palmar fascia of patients undergoing carpal tunnel surgery (CT). Cells were maintained in MEM- α -medium supplemented with 10% fetal bovine serum along with 1% antibiotics. All cultures were used at passage levels between 3-5 with no changes evident in cell morphology.

In-Vitro Wound Healing Assay

Cell cultures from all the three groups (CT, PF and DC) were plated on 6-well plastic dishes and grown to confluence in α -MEM-medium supplemented with 10 % FBS. The cells were quiesced for 48 h in the media containing 0.1 % dialyzed FBS. An *in vitro* wound healing assay was performed in near confluent cultures. Cells were scraped making an ~1-cm wide denuded area, then either stimulated with PDGF-BB (2 ng/ml) or forskolin (10 μM) or Ro20-1724 (10 μM) or with control vehicle only. Photographs were taken at 0 h and 48 h and the relative distance traveled by the cells at the acellular front was determined by computer-assisted image analysis. Markings on the plates ensured measurement at the same site of the photographs at each time point.

Immunoblotting and Immunoprecipitation

Phosphorylation and activity of RhoA was assessed by immunoprecipitation and immunoblotting. For immunoprecipitation, DC - derived fibroblasts were treated with forskolin (10 μM) for 30 min before treating with PDGF (2 ng/ml) for 10 min. Cell lysates were incubated overnight at 4°C with the indicated antibody, mouse monoclonal Active RhoA (New East Biosciences), or rabbit Anti-RhoA (phospho S188) (abcam). Immunocomplexes were incubated with protein G-agarose and centrifuged. The pellets were washed and precipitated proteins were size-fractionated by SDS-PAGE and transferred to a PVDF membrane. RhoA phosphorylation and RhoA activity were determined by immunoblotting using the mouse Anti-RhoA (abcam).

Results

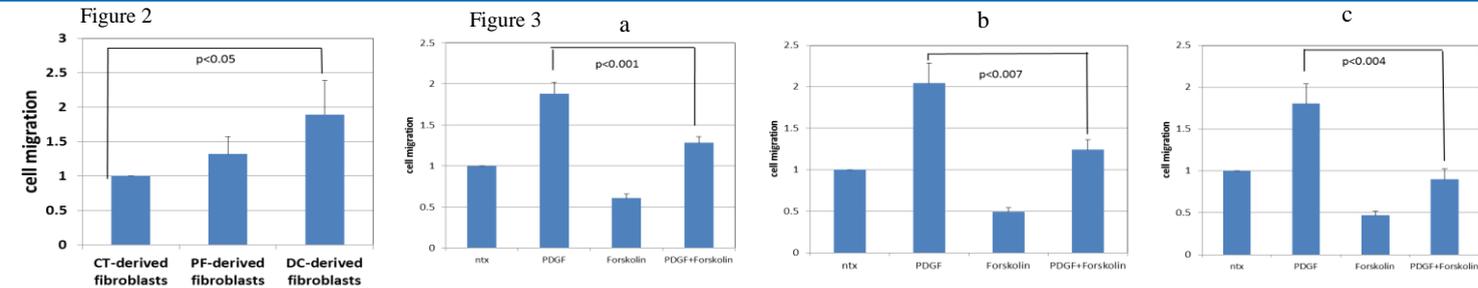


Figure 2: DC-derived fibroblasts exhibited increased basal cell migration in comparison to CT- and PF-derived fibroblasts: An *in vitro* wound healing assay was performed on 6 independent primary cultures derived from CT-, DC- and PF-tissues to determine the migratory efficiency. The values are mean \pm SEM of two independent experiments performed in triplicate. Statistical analyses were performed by Student's t-test. CT-carpal tunnel derived fibroblasts; DC-Dupuytren's derived fibroblasts; PF-palmar fascia derived fibroblasts.

Figure 3: Forskolin inhibition of PDGF-stimulated cell migration was more pronounced in DC-derived fibroblasts.

Six independent cultures derived from (a) CT-, (b) PF- and (c) DC-tissues were grown to near confluence and were quiesced for 48 h and were left untreated or were stimulated with forskolin (10 μM) in the presence or absence of PDGF (2ng/ml). An *in vitro* wound healing assay was performed and photographs were taken at 0 h and 48 h and the relative distance traveled by the cells at the acellular front was determined by computer-assisted image analysis. Values are mean \pm SEM of two independent experiments performed in triplicate. Statistical analyses were performed by Student's t-test.

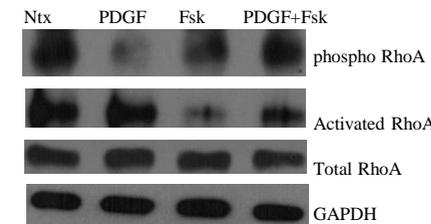


Figure 4: Elevated cAMP levels decreased PDGF-induced RhoA activity by RhoA phosphorylation in DC-derived fibroblasts: DC-derived fibroblasts quiesced for 48 h were treated with forskolin (10 μM) for 30 min before treating with PDGF-BB (2 ng/ml) for 10 min. Cells lysates containing same amounts of proteins were immunoprecipitated with anti-active RhoA and anti-phospho RhoA. Then immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with anti-RhoA antibody. GAPDH was used as loading control. Representative blots of two independent experiments are shown.

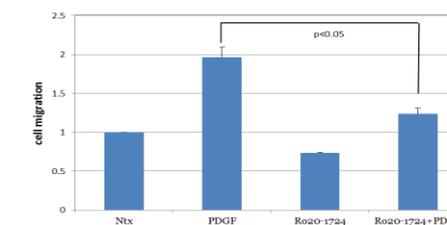
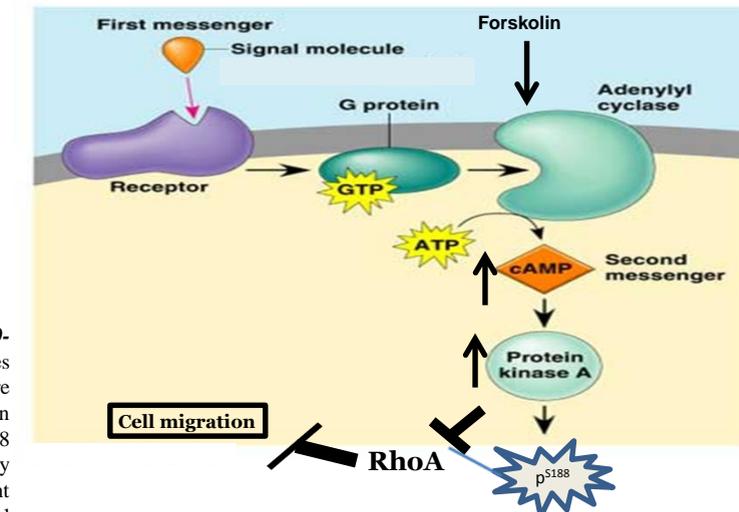


Figure 5: PDGF-induced cell migration was inhibited by the PDE-4 inhibitor Ro20-1724 in DC- derived fibroblasts : Two independent cultures derived from DC-tissues were grown to near confluence and were quiesced for 48 h and were left untreated or were stimulated with Ro20-1724 (10 μM) in the presence or absence of PDGF (2 ng/ml). An *in vitro* wound healing assay was performed and photographs were taken at 0 h and 48 h and the relative distance traveled by the cells at the acellular front was determined by computer-assisted image analysis. Values are mean \pm SEM of two independent experiments performed in triplicate. Student's t-test was used to evaluate the statistical significance.



Summary

In sum, we show that increase in cAMP levels can reduce PDGF-induced cell migration by inhibiting active RhoA.