# Investigation of the effect of POMC peptides on dermal fibroblast migration and its role in wound healing

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Running title:**Enhanced fibroblast migration by POMC peptides**
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## Abstract:

The human skin contains several different fibroblast subtypes including dermal sheath (DS), dermal papilla (DP) and inter-follicular dermis cells (DF). It has been shown that quicker healing and less scar formation is related toproportionally fewer numbers of DS and DP fibroblasts. TGF-β plays a crucial role in wound healingby attracting fibroblasts and promoting their synthesis of collagens I, III, and V, proteoglycans, fibronectin and other extra cellular matrix (ECM) components; and that the POMC peptides α-MSH/ ACTH may ameliorate hypertrophic scaring by antagonizing TGF-β. The aim of this project was to assess the role of POMC peptides α-MSH/ ACTH in modulating migratory activity of fibroblasts *in vitro* using scratch assay with or without POMC peptides and the transwell assay in the presence of the extracellular protein fibronectin. Transwell assay showed that fibronectin increased fibroblast sub-types migration with DS cells migrated through fibronectintranswells more effectively than other fibroblast sub-types. POMC peptides, ACTH induced the greatest increase in migration with fibronectin coated wells. In addition, scratch assay showed that the fibroblast migration was significant in the presence of the POMC peptides.

## Introduction

Skin is complex, multilayered organ, which include appendages (hair follicles, sebaceous glands, sweat glands, apocrine glands) and consists of mixed cell types and extracellular components and contains three layers: epidermis, dermis and hypodermis (Freinkeland Woodley, 2001). The epidermis is highly organized kinetic structure in which cells divide, move and differentiate and contains many cell types including keratinocytes, melanocytes, Langerhans cells and Merkel cells. The dermis is the connective tissue component of the skin, which is much thicker than the epidermis and composed primarily of fibroblasts, collagen, elastin, and adipocytes (Slominski, et al, 2000a&b; MacNeil 2007). It protects the body from mechanical injury and provides its elasticity and tensile strength. There is great interest in

fibroblast regulation because improved activity is important for wound healing and during formation of hypertrophic scars (Freinkeland Woodley, 2001). The dermis and epidermis interacts together to maintaining the properties of both tissues and in repairing the skin in the case of trauma. In human beings there are clear differences in wound healing responses between hairy and non-hairy body sites (Jahoda, et al, 2001a). The contribution of the hair follicle derived dermal cells results in qualitatively enhanced dermal repair (Jahoda et al, 2001b). Normal wound healing involves complex and dynamic events leading to the repair of injured tissuesincluding rapid proliferation of fibroblasts, followed by collagen biosynthesis (Montagna. et al, 1964). Human skin contains several different fibroblast subtypes including inter follicular dermal fibroblasts (DF), the follicular dermal sheath fibroblasts (DS), and the follicular dermal papilla fibroblasts (DP). The dermal sheath cells maintain and regenerate the dermal papilla, a key for hair growth; in addition these cells may have other roles in the wound healing and repair of skin dermis after injury (Jahoda, et al, 2001b). Fibroblasts degrade the provisional matrix via metalloprotease (MMPs) and respond to cytokines by proliferating and synthesizing new extra cellular matrix (ECM) to restore the wounded tissue with a connective tissue scar. Transforming growth factor TGF-β contributes to the scar development by attracting fibroblasts and promoting their synthesis of collagens I, III, and V, proteoglycans, fibronectin and other ECM components. TGF-β concurrently inhibits proteases while enhancing protease inhibitors, favouring matrix accumulation (Wahl, 1999;Branton and Kopp, 1999). The fibroblasts are stimulated to migrate into the wound area, proliferate and produce the matrix proteins fibronectin, hyaluronan (HA) and later collagen and proteoglycans. Once within the wound environment, fibroblasts proliferate and establish to create the new ECM, which is important for the repair progression and supports more ingrowth of cells (Grinnell, 1994). Fibroblasts differentiate into myelofibroblasts which help the wound begins to contract (Eichler, et al, 2006;Romo, et al, 2006),contraction can last for several weeks (Mulvaney, et al, 1994) and continues even after the wound is completely re-epithelialized. Myofibroblasts are stimulated by growth factors and ECM fibronectin to move along it linked to fibrin in the provisional ECM in order to reach the wound edges(Hinz, 2006). The proopiomelanocortin (POMC) peptides ACTH, Alpha-melanocyte stimulating hormone (α-MSH), and ß-MSH and ß-endorphin peptides are detected in normal and

pathological cells as melanocytes, keratinocytes, Langerhans cells, and mononuclear dermal inflammatory cells by immunocytochemistry. The human dermal endothelial cells and fibroblasts produce and released a-MSH and ACTH immunoreactivity into the medium (Slominski, et al, 2000a; Millington, 2006). α-MSHis the most potent melanotropic peptide arising from the proteolytic cleavage of POMC, and is produced in the skin. Moreover, the melanocortin receptor 1 (MC-1R), which is bound by α-MSH and ACTH, is expressed on some types of cells in the skin as keratinocytes, dendritic cells, macrophages and endothelial cells **(** Luger, et al, 2000). α-MSH peptides and its receptors regulate melanogenesis but can also affect non-pigmentary processes, such as inflammation, apoptosis and sebogenesis (Ruta, et al. 2007). Theyhave effect on the regulation of immune and inflammatory responses, hair growth, exocrine gland activity and extracellular matrix composition (Luger, et al, 1995). Cytoprotective function for this hormone or peptides in defence of the skin from exogenous factors or stress e.g. would occur following UV exposure or exposure to agents inducing inflammation or oxidative stress (Eves, et al, 2000). Production of melanocortins and expression of their receptors suggest a complex autocrine and/or paracrine regulatory system whose disorder always affects skin homeostasis. These effects of the melanocortin are mediated by melanocortin-1 receptors which are most ubiquitously expressed by human skin cells (Böhmand Luger, 2000). Alpha-MSH is a basic tridecapeptide,the amino acid sequence of α-MSH is identical to the 1-13 (N-terminal) amino acid sequence of ACTH although α-MSH has slight straight control on glucocorticoid secreted from the adrenal gland (Catania, et al. 1993). α-MSH originate primarily in the pituitary, but it is also secreted in low concentrations in the central nervous system, the skin, and other sites. This peptide was named for its effect on pigmentation in amphibian skin; although it is important to other functions due to its tissues distribution in higher organisms (Catania, et al. 1993).

## Methods and materials:

**Fibroblast cellsculture and maintenance**

Fibroblast cells were maintained in growth medium,containing 500 ml RPMI 1640, 50ml of 10% Foetal calf serum, 5ml of Penicillin/streptomycin and 5ml of L-glutamine at an appropriate temperature and gas mixture (5% $CO_2$, 95% Air at $37^0$C), in cell incubator in humid atmosphere.

## Immunocytochemistry:

For immunohistochemistry fibroblastscells ($5x10^3$ cells/well) were allowed to attach overnight in the incubator then washed with PBS, drained and incubated in ice-cold methanol for 10 min at $-20^0$C. The cells were then fixed in 4% paraformaldehyde in PBS for 10 min at room temperature (this was used for the melanocortin 1 receptor as this receptor is located on the cell surface). The cells were drained and washed by PBS 200 µl/well, 3 times for5 minutes. Following incubation with 200 µl of 10% Goat serum (GS) for 90 min to block any non- specific sitescells were washed three times in 200 µlPBS for 5min then incubated with 100 µl of diluted primary antibody (Table 1) per chamber overnight at $4^0$C. After washing in PBS, 100 µl of pre diluted secondary antibody per chamber was added, and incubated for 15 min. The cells washed with 200 µl PBS for 5 min, three times and drained. 100 µl of streptavidin peroxidase solution was added to the cells and incubated for 20 minutesafter washing three times with 200 µl PBS for 5 min, 200 µl of distilled water was added and incubated for 2 min then 200 µl of chromogenwas added in each well and incubatedforfurther 10 min (the negative control was used to check for non-specific binding and to determinate duration exposure) which result in a red precipitate at the antigen site. During the incubation period the progress of the reaction was monitored under bright field of light microscop. The chambers were washed with distilled water 200 µl per chamber, to stop the chromogenreaction. After mounting with pre warmed glycerol and applying cover slip, pictures were taking of the cells visualize the type and location of any staining.

Table 1: primary antibodies plating of cells onto 8-well glass chamber slides

| Antibodies | Concentration |
|---|---|
| Fibroblast marker | 1 : 200 (positive control) 1 : 500 1 : 1000 |
| Melanocortin-1 receptor | 1 : 10 |
| Vimentin | 1 : 100 1 : 400 |

**Fibroblasts Migration test: The "Scratch Assay"**

For scratch assay cells were seeded in 24-well plates (Nunc, Langenselbold, Germany) at 3x $10^4$ cells/well,at 80% confluence, a scratch wound using a sterile pipette tip was performed and the size of the gap was measured; cells were incubated with 500 µl of working dilution POMC peptides

(a-MSH, ACTH), each at a concentration of $10^{-7}$ M and $10^{-8}$ M , were added to the cells, 500 µl of full media and basal media were added to the cells as appositive and negative controlled respectively and the closing of the scratch wound was measured for 72 hours or until wound was closed.The cells were then stained with (Toluidin blue 1% in Borax) at room temperature, tofacilitate digital photography of the wound edges under low magnification usingcolour digital camera (Nikon coolpix) connected with the light microscope.

**Trans-well Migration of the Fibroblasts in presence or absence of Fibronectin:**

To assess cell migration fibroblasts were placed in the top chamber of transwell migration chambers (8 or 5 Am; BD Biosciences, San Jose, CA).The lower chamber was filled with 500 mL of either conditioned medium or control medium with or without 50 µlcontaining 5µg/$cm^2$offibronectin. The fibronectin solution was aspirated from the transwell plates on the second day. Then the cell suspension was prepared and 8 x $10^3$ cells/ well was seeded gently in to the transwells after the counting. 150 µl of cell suspension was added to each transwell, and 500 µl of the conditioned media (α-MSH, ACTH) as a chemo-attractant, the full media, and the basal media, were added to the bottom of each well. Cells that had not migrated to the lower chamber were removed from the upper surface of the transwell membrane with a cotton swab. Migrating cells on the lower membrane surface were fixed, stainedusing Methanol/acetone containing 0.1% crystal violetand Hoechst 33258 dye, and photographed.

## Results

Transwell Assay:

In transwell assay the subpopulation of the fibroblast (dermal papilla, dermal sheath, interfollicular cells types) from the same individual (male 46 years old) were assessed and used at density of 8x$10^3$ cells per well. The chemoattractant factor fibronectin secreted by the fibroblast cells affects the cells migration, and was investigated in this project. It was shown that fibronectin was a potent chemotactic signal for migration. Figure 1 showed increased the migration rate of DS fibroblast cells with the fibronectin compared with those without fibronectin.

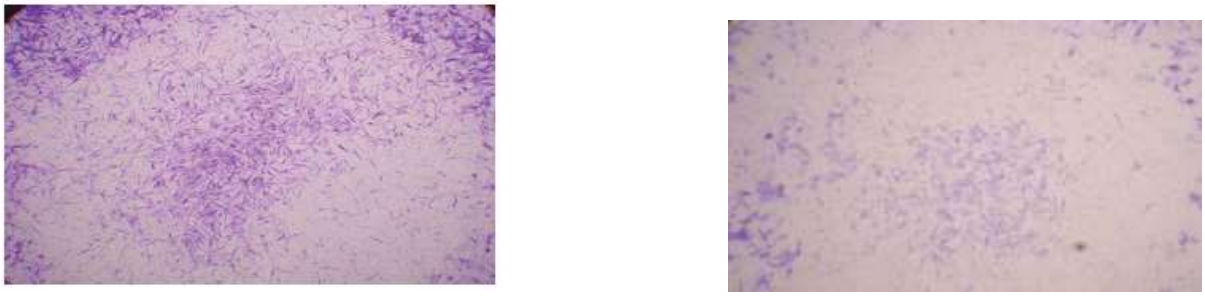


**Figure 1:** DS fibroblast migration through the membrane coated by fibronectin (a) or un-coated by fibronectin (b) in full media (serum supplement) X2 objective magnifications.

a. with fibronectinb. without fibronectin

## Effects of the POMC peptides on fibroblasts migration:

It was also investigated how fibroblast migration is affected by the addition of POMC peptides (α-MSH / ACTH) as a chemotactic stimulus for fibroblasts, results showed that there increase of migration of cells grown on wells containing α-MSH/ ACTH peptides (Figure 2 and 3).

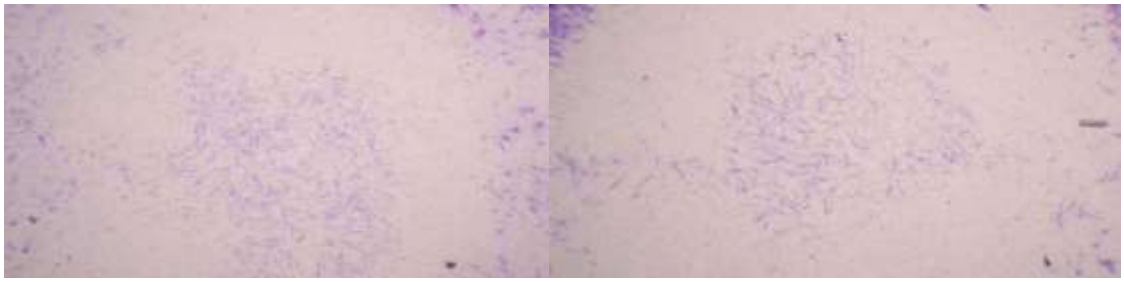


**Figure 2:** DS fibroblast migration through the membrane coated by fibronectin in basal media (serum starved) containing α- MSH 10-7M. X2 objective magnifications.

a. α- MSH $10^{-7}$M with fibronectin b. α- MSH $10^{-7}$Mwithout fibronectin

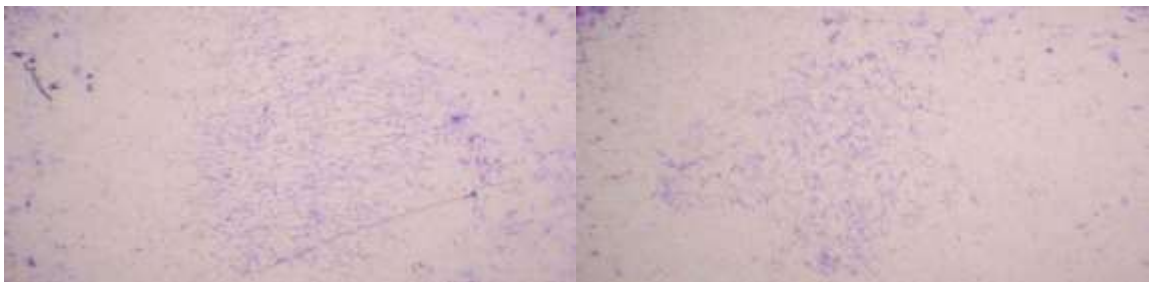


**Figure 3:**DS fibroblast migration through the membrane coated by fibronectin in basal media(serum starved) containing ACTH $10^{-7}$ M peptide in the presence of fibronectin. X2 objective magnifications

a. ACTH $10^{-7}$ Mwith fibronectin b. ACTH $10^{-7}$ Mwithout fibronectin

## Scratch Assay:

The rate of wound closure and cellular migration of the fibroblast cells wasimportant because if follicular fibroblasts are involved in the wound healing itwould be useful to discover their potential rate of migration into a zone of injury. Theaverage distance between leading cell edges photography

of mechanical scratcheddistance of approximately 2.0mm, at t0, then after 72 hours from the scratch (t72), andthen after 144 hours from the scratch (t144).

**Figure 4:** Typical central score created on the floor of 24- well plate onto a monolayer of fibroblast, at seeding density of 3x104 /well. The photographs were taking after the mechanical scratch directly at (t0), by digitalcamera (A camera 1/15, F3.2), x 2 objective magnification.

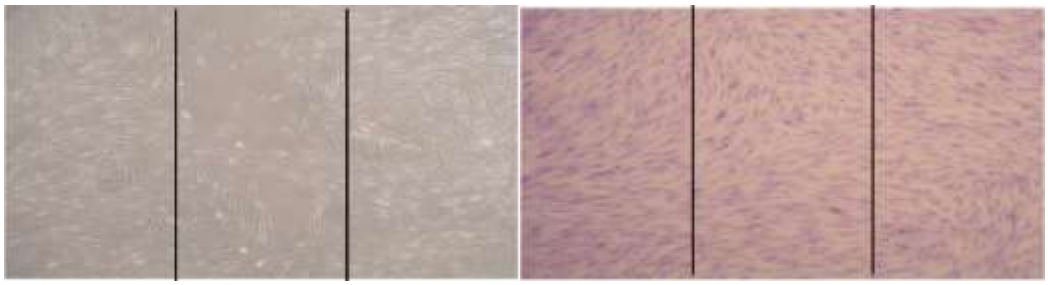


**Figure 5:** Fibroblast migration at (t72) and at (t 144) with fullmedia (serum supplement), x4 magnification

a. at (t72) b. at (t 144)

The rate and extent of cell movement was greater with the basal media containing POMC peptides than with basal media alone.Moreover, the migration of fibroblasts was greater in the present of ACTH peptide than in the present of α-MSH peptide (Figure 6).

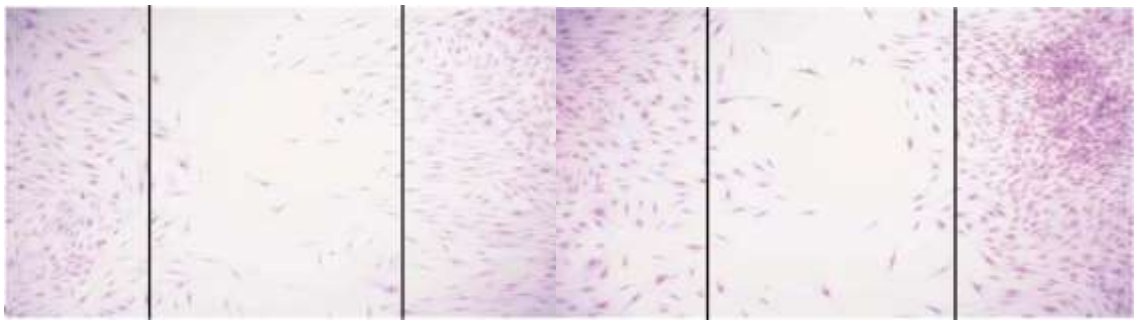
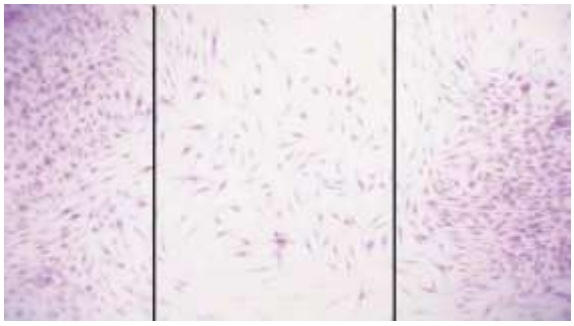


**Figure 6:** Fibroblast migration at (t 144). (a) Basal media only. (b) Basal media with α-MSH.10-7 M, (c) basal media with ACTH 10-7 M. X4 objective magnifications.

a. Basal media only b. Basal media with α-MSH.10-7 M



c. basal media with ACTH 10-7 M

## Immunocytochemistry

It was important to know about the immmunocytochemistry of the fibroblast cells *in vitro*, to find out the localization of proteins in the three fibroblastsubtypes cells, within chemically fixed cells, primary antibodies that identify proteins were added andincubated with the cells, and detected by fluorescence or colorimetric process andvisualized by microscopy. The immunocytochemistry was done for the interfolliculardermal fibroblast (DF) cells for female 54 years, and also for fully matched cell types(DF/ DS/ DP) of the male 46 years, but for many reasons there was insufficient number ofthis cells, as the cells become contaminated at the beginning of the cell culture and thenthe human fibroblast cells enter stage of arrested (no more growth) so the female 54 was used.

In contrast with the positive control (fibroblast marker antibody 1:200) the fibroblastcells were tested positive for the protein vimentin (positivestaining) with the twoconcentrations (1:500, 1:1000) it is giving the red colour after adding the chromogen.

The cells also exhibits the melanocortin-1 Receptor (MC1R) expression atconcentrationof (1:10) only, but did not reveal any staining of (1:25/1:50/1: 100).The results areshows in figure x 10 objective magnification.

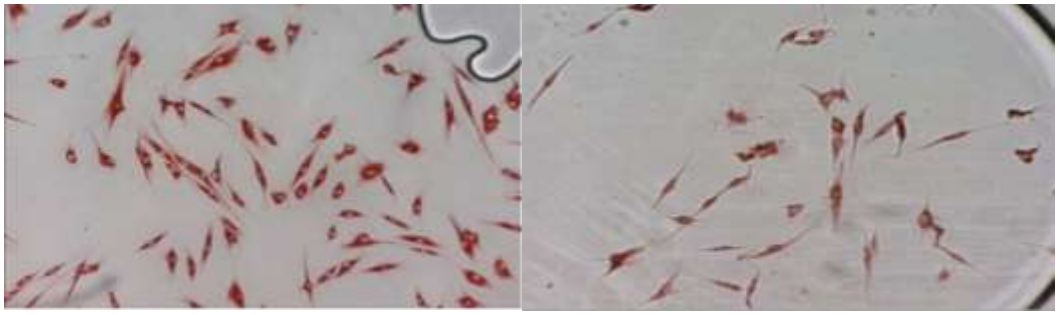


**Figure 7**: Immunocytochemistry staining of dermal fibroblast for fibroblast marker (a. 1:200; b. 1:500), x10 objective magnification under phase contrast.

a. 1:200 b. 1:500

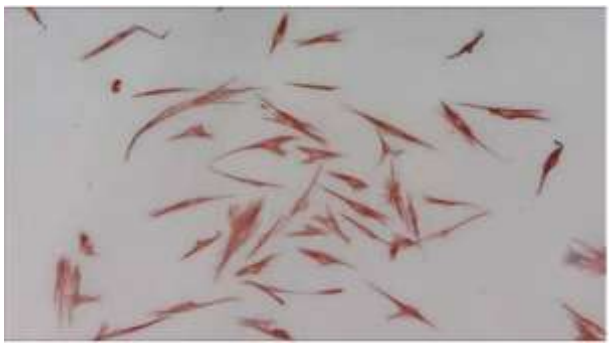


**Figure 8:** Immunocytochemistry staining of dermal fibroblast for Vimentin (1:400), x10 objective magnification(Staining intensity: +++ high).

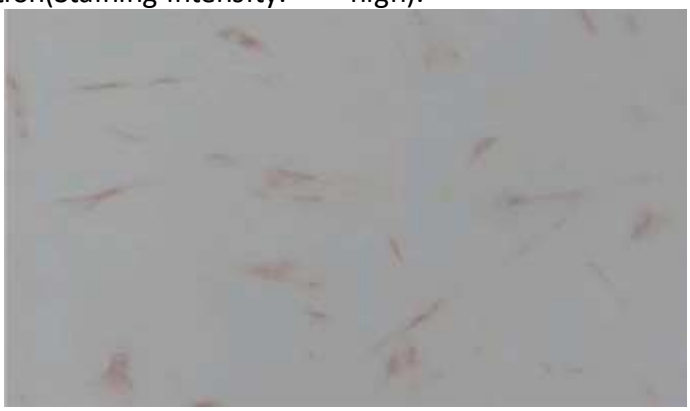


**Figure 9:** Immunocytochemistry staining of dermal fibroblast for Melanocortin 1receptor(1:10), x10 objective magnification(staining intensity ++medium).

## Discussion:

Fibroblast cells proliferate, migrate, synthesize ECM macromolecules, secretegrowth factors, they are professionals in movement, ECM production, and deposition,and in a multitude of filopodial activities that enable them to shape the ECM and pullother cells. They make collagens, glycosaminoglycans, reticular and elastic fibres,and glycoproteins found in the extracellular matrix andtissue damage stimulates them and induces theirproliferation. Stimulation of fibroblast migration by cytokines promotestheir migration and enhances the rateof wound healing (Schreier, et al, 1993). This study provides strong supporting evidence for an active involvement of thefibroblast cells, particularly DS cells in the wound healing. Using the transwell assay, human fibroblasts shows high rate of movementthrough the 8 µm pore membrane, this movement observed specially when combinedwith extracellular matrix protein (Fibronectin), but it is also significantly observed whencombined with POMC

peptides. Cell migration can be regulated by peptides to ensure a fast formation of new connectivetissue at the wound site. After the mechanical scratch of the monolayer, cells moveforwards, co-ordinately as a sheet, to fill up the space without the requirement for celldivision (Hall, 2005). The scratch assay shows successful results, revealed asignificant high migration capacity of human fibroblast cells in to the injured area. Therewas some variation in the cellular migration according to the media and thechemoattractant substance (POMC peptides). The enhanced healing or closure of thescratched area noted following the addition of POMC peptides, but the more potentchemotactic agent is the serum followed by the ACTH 10-7 M.. Itcan be seen that in thefirst 72 hours from the scratch applied there is rapid rate of cellsmigration, the cellsbecomes retracted at the wound edge, re-spread and extend filopodia intothe scratchspace, where they are in contact with their neighbours. Interestingly at 144 hours fromthe scratch wounding (the $7^{th}$ day)there was significantly faster migration of the cells (leading to injury closure) i.e. as in monolayer feed with supplement medium. The fibroblasts express the intermediate filament protein vimentin which is the earliest tobe expressed during cell differentiation, a feature used as a marker to distinguish theirmesodermal origin. Vimentin is secreted in a large range of mesenchymal cell types includingfibroblasts,endothelial cells etc, and in a number of other cell types derived frommesoderm andmesoderm derived epithelia. Vimentin isfound in various different tumours but is particulary expressed inthose originated frommesenchymal cells. Toinvestigate that it was important toknow about the Immunocytochemistry of thefibroblast cells in vitro, the fibroblast cells weretested positive for the protein vimentin. The POMC peptides products α-MSH and ACTH bind to specific receptors known as themelanocortin (MC) receptors. There is increasing evidence that the MC receptor type1 (MC-1R) is expressed in vitro by fibroblasts as well as by several other cutaneous celltypes besides melanocytes and keratinocytes (Böhmet al, 1999). The fibroblast cellsin vitro were positive for MC1R. The MCR subtypes havebeen individually expressed in mammalian cell lines and their binding to MSH peptideshas been characterized. Site-directed mutagenesis and molecular modelling of the MC1R has revealed a putativeinteraction of individual amino acids in the receptor with theMSH ligands. In conclusion, results presented in this work provide that the fibroblast sub-typesDS play a key role in the wound healing specially when stimulatedby POMC peptides. It was concluded also that the extracellular matrix protein(fibronectin) increased the migration rate of the fibroblasts.

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