ORIGINAL ARTICLE

# THE ISOLATION OF HUMAN SENESCENT CELLS BY THEIR INABILITY TO ATTACH TO FIBRONECTIN

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**ABSTRACT**

*Senescence derived from a Latin word senex means old age. Senescence in an organism's life is a phase of developmental decline, and a loss of replicative capacity in cell culture. Cellular senescence is due to cycle arrest in the G1 phase of the cell cycle in response to physiological levels of mitogens. Senescence is often associated with DNA Damage foci and it involves the cell cycle inhibitors p21WAF and P16INK4A together with other proteins, including senescence-associated b Galactosidases (SA-bGal) and several cytokines.*

*The objective of the study was to test whether the senescence of normal human epidermal keratinocytes (NHEK) or the initiation of NHEK differentiation with, or without stratification, leads to reduced adhesion to FN.*

*NHEK underwent three different adhesion experiments and at three different calcium (Ca) concentrations, starting from 0.09mM Ca and then 0.6mM Ca for 5 days and 1.0mMCa for 24 hrs. At all these concentrations cell adhesion time courses were done. The experiments showed that senescence slowed the rate of adhesion of NHEK cells to FN. Also at 0.6mM Ca NHEK differentiation alone did not influence the rate of adhesion to FN, but differentiation along with stratification at 1.0mM Ca did influence the adhesion rate. Ki67 immunofluorescence staining revealed that whilst the senescent and stratified NHEK cultures showed reduced numbers of cycling cells the differentiated cells in 0.6 mM Ca did not. Unlike the situation with oral fibroblasts, we were unable to demonstrate a large enrichment for senescent NHEKs in the floating population at any time point as assessed by SA-bGal staining of the different cell populations.*

*It was concluded that both the later stages of terminal differentiation (stratification) and senescence reduced the adhesion rate of NHEKs to FN but that differentiation alone did not. The reduced rate of adhesion to FN was associated with a reduced number of cycling cells.*

*The inability to enrich for senescent NHEKs using the rate of adhesion to FN may be related to the confounding influence of NHEK terminal differentiation on FN adhesion rates.*

**Key Words:** *Senescence, Senex, NHEK, Fibronectin, Queen Marry, Khyber college of Dentistry.*

## INTRODUCTION

Ageing or senescence is a major cause of disease, suffering and death. The history of senescence starts with the discovery of the Hayflick limit in 1961. Hayflick first started his experiments in the Wister institute, Philadelphia on human foetal lung fibroblasts and found out that cells stop dividing after 40 to 60 cycles and then senesce. Telomeres shorten with each round of cell division. When the telomeres of some of these chromosomes become shorten, they lead to cell cycle arrest, which is due to the inactivity of telomerase.[1] These chromosome ends are protected by telomeres. According to telomere hypothesis of senescence the progressive shortening of telomere is a clock mechanism that occurs in normal somatic cells with each round of cell division.[2] In young cultures senescent cells showed mitochondrial dysfunction and telomere shortening and DNA damage in telomeres. These results suggested that the Hayflick limit is only applicable to cell line with mass population and that life span of an individual cell is governed by telomere shortening due to oxidative stress.[3] In studies on human patients cells were taken from subjects and *in vitro* studies cells undergo premature senescence.[4]

It was found that cells senesce in young age in atherosclerosis and was found in Werner and Progeria

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syndromes. On the other hand studies were done on dyskeratosis congenital and ataxia telangiectasia and it was found that these patients did not show atherosclerosis. Cellular senescence affects organs and tissues in the body as they lose their functions due to growth arrest of the cells so in this case liver cells also senesce and liver is damaged along with the haematopoietic compartments.[5] As these cells reach their peak and senesce they lose their integrity and function and as the cells stop regenerating, ageing ensues and this is ageing phenotype. The adhesion of cells is critical to many biological processes including embryological development, tissue repair, differentiation, immune response and malignant transformation. The attachment of cells to the extracellular matrix or to their surroundings is very important because it maintains proper cellular function.. Adhesion of cells to the ECM involves receptors which are cell surface receptors. Most of these receptors belong to integrin receptor family. These receptors make a link between the ECM and cytoskeleton structures and these transmit signals bidirectional.[6]

Many researchers have done a lot of work on fibroblasts and keratinocytes and how they attach to the ECM. Human epidermal keratinocytes retain many of their properties as they are grown in culture.[7] *In vitro* structure the keratinocytes differentiation and stratification is also controlled by the Ca ions concentration.[8] One of the cell to cell adhesion protein is cadherin. It is a family of glycoproteins which influences the cell surface integrins levels of keratinocytes.[9]

In the present study senescent cells were isolated from cells population in culture by their inability to attach to FN. The study will help the histopathologist to understand the role calcium ions in senescent cells. And will help in the future prevention and management of various pathologies related to senescent cells.

## METHODOLOGY

NHEK cells were prepared by centrifugation at 800rpm after the cells were transferred to the culture dish these cells were sub cultured by adding medium and transferred to their culture plates. Cultures were fed twice a week and are used as needed for experiments. Keratinocytes were sub cultured not more than 8 days after being plated. Senescent cells in culture increase steadily as the primary line approaches the end of its replicative life span, therefore after each sub culturing; the cells were counted and plated according to the desired densities.

The cells were cryopreserved when they were not needed by centrifuging and then cryopreserving in 1ml DMSO (Sigma Chemical Co., Poole Dorset, UK) and 9ml of serum-containing DMEM so the final concentration on DMSO was 10% vol/vol. The cells were diluted in this medium, mixed well and liquated into plastic tubes before storing at -80°C for at least 24 hr before shifting to the permanent liquid nitrogen storage facility (-196°C).

The NHEK cells were adhered to the fibronectin . The adhesion experiments were performed preparing the cells in the culture plates and later stained to detect the senescent cells for the experiment to start.

### Experiment 1. The effect of 1mM Ca on NHEK adhesion to FN versus 0.09Mm Ca on NHEK adhesion to FN

In this experiment, 2 groups are taken. 1 group which is the high (1.0mM) Ca group where 91µl of Ca is added to 20ml of medium, old medium from the culture plates is replaced with this high Ca medium 10ml in each plate and the cells are allowed to grow for 24 hours in the incubator. This manipulation allows the NHEKS to both differentiate and stratify[10], the latter of which involves detachment of the NHEKs from the extracellular matrix of the basement membrane containing FN.

After 24 hrs when the cells are grown these cells are thawed and dilutions are made and adhesion experiment is performed on 6 FN-coated plates and 6 non FN coated plates. After the time course is done, the next day SA-βGal staining is performed and the following day counter stained with nuclear fast red.

### Experiment 2. The effect of 0.09mM Ca versus 0.6Mm Ca for 5 days on keratinocytes adhesion to FN.

Again the same experiment was performed, cells were grown in 0.6Mm Ca for 5 days, which allows differentiation without stratification.. 51µl of calcium (Ca) was added to 20ml of SFM medium to give a final concentration of 0.6mM and 10ml of medium each was replaced with the old medium in each culture plate. The adhesion time course experiment was performed on these cells, followed by SA-βGal staining and counter stain as above.

### Experiment 3. The effect of senescence on the adhesion of NHEKs to FN

This time senescent cells were passaged and allowed to grow in SFM medium in culture plates until they did not double in number for two weeks. The adhesion time course experiment was performed on these cells, followed by SA-βGal staining and counter stain as above.

### 3.3: Ki67 staining

To test whether any of the above manipulations have any effect of cellular proliferation we stained for the Ki67 antigen. Ki67 antigen is a nuclear protein which is associated with cellular proliferation. Young NHEKs at passage 3 (Y) in 0.09Mm, 0.6 mM and 1.0mM Ca, and senescent NHEKs at passage 9 (S) in 0.09 mM Ca were stained and compared.

## RESULTS

In the adhesion experiments, NHEK cells were used, to see the effect of these cells and their attachment to FN at different time intervals starting from 0 time to 50 mins. Different Ca concentrations were also used to see their ability of differentiation and stratification functions. NHEK cells were compared with 0.6 mMCa and 1.0mM Ca concentrations and these cells were compared with 0.09 mM Ca in the normal SFM medium, as shown in the graphs in Fig 1(a,b), 0.09 mM Ca NHEK cells are compared with 0.6mM Ca concentrations after 5 days and their ability to attach to FN. It showed that at 0 time almost 50% of the cells adhered to FN and almost 50% were floating and at 50mins almost 85% of cells adhered to FN with only 20% of floating cells at this time, which implied that there was no remarkable difference among the 2 Ca groups at 0.6mMCa.

All these experiments were carried out three times at the same Ca concentrations and each time maximum cells adhered at about 30 mins and 50 mins. The SA-βGal results also showed that at 0.6 mM Ca concentration the percentage of cells stained were higher as compared to 0.09 mM Ca, at 1.0 mMCa 100% cells adhered to FN. The SA-βGal activity at 1.0 mM Ca showed that 40 to 50% cells adhered that means that adherence of cells was increased with increase in Ca concentrations. The mean (±) S.D for the three experiments on each Ca concentrations was calculated to determine the variability of the data (Fig 2). Ki67 staining was also performed to show senescent cell in the cell cycle phase as shown in Fig 3.

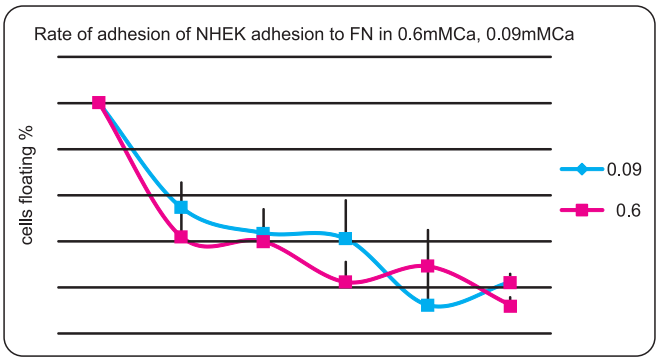


Fig 1: Illustrates the effect of NHEK adhesion after 5 days in 0.09mMCa or 0.6mMCa (floating)

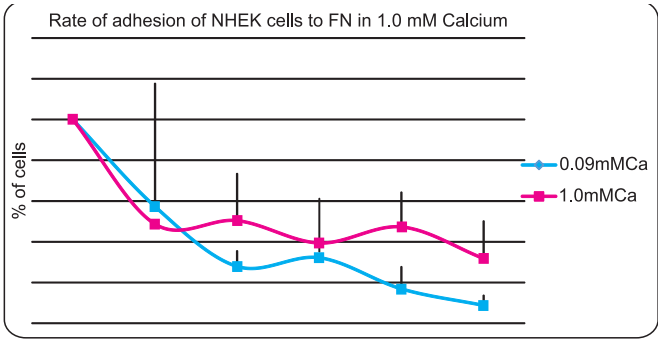


Fig 2: Illustrates the effect of 1.0mMCa adhesion of NHEK cells to FN

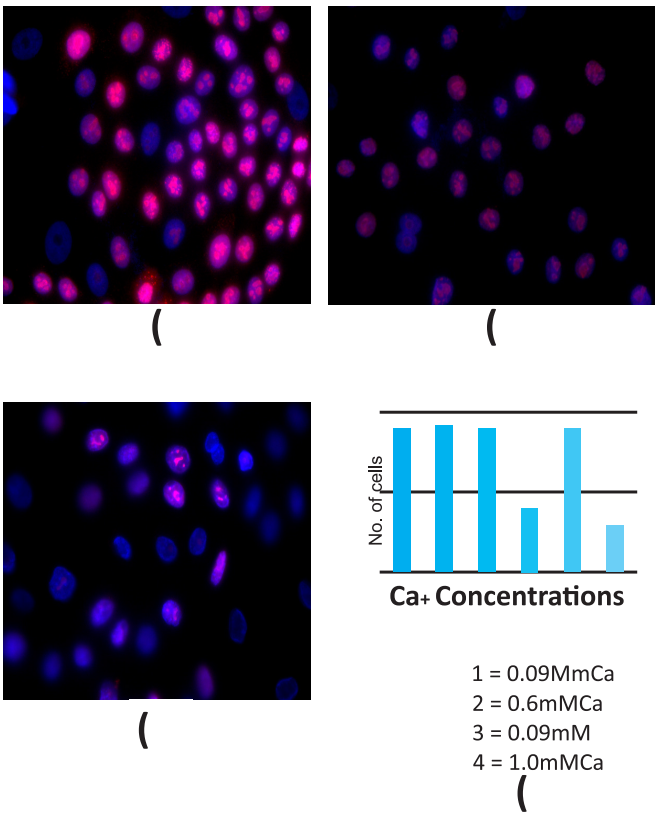


Fig 3: Illustrates the Ki67 staining at different Ca concentrations and following senescence

## DISCUSSION

Senescence is the result of cellular stresses, including DNA damage, that give rise to a cell cycle arrest in the G1 phase of the cell cycle; it involves the accumulation of the cell cycle inhibitor $p16^{INK4A}$.and a collection of other proteins, including SA-βGal, whereas differentiation does not. However, in both situations, the cells fail to enter the S phase in response to physiologic mitogens.[11] Once the cells have entered a state of senescence, numerous genetic changes take place including cyclins, CDKs and the cyclin – dependent –kinase inhibitors [12] the cell cycle check points are controlled by p53 and pRB proteins; the tumour suppressor genes and disruption in this pathway due to DNA damage responses or other

factors leads to replicative lifespan and crises.[13] A lot of work has been done on fibroblasts and keratinocytes and their attachment to FN. Cell –cell adhesion is controlled by different calcium concentrations and how the differentiation and stratification take place. Previous studies by Owens showed that different calcium concentrations regulate differentiation and stratification independently. At 0.03mM Ca the keratinocytes form only transient cell-cell adhesions and show low levels of differentiation but at 0.6mM differentiation can occur without stratification. However, raising the calcium concentration to 1.0mM resulted in stratification as well as differentiation and stratification is generally held to involve the reduction in the affinity of the extracellular matrix receptors, the integrin for their ligands, which include FN.[10] in the present study it was first investigated whether the cells exited the cell cycle in these different calcium concentrations by staining the cultures with Ki67, an antibody specific for cells in cycle. It showed that keratinocytes in 0.03 and 0.6 mM Ca had more than 90% of their cells in cycle, whereas those in 1.0mM Ca had only about 40%. This suggests that only when keratinocytes lose the ability to attach to the basement membrane do they begin to exit the cell cycle. Next it was tested whether late passage keratinocytes had also exited the cell cycle and found that in this case also the Ki67 index fell to 30%. This may also explain why even in early passage cultures the Ki67 labelling index is not 100% because even early passage cultures of normal human cells contain some senescent cells. Joseph M and Watt F M, worked on keratinocytes :stem cells and cells which undergo terminal differentiation and found out that β1 integrin expression helps in distinguishing stem cells from those undergoing terminal differentiation *in vivo* and *in vitro*.[14] In this study NHEK cells at early and late passage were investigated. Also these cells were subjected to different Ca concentrations; the controls were cultured in 0.09mM Ca, which was the normal Ca concentration in serum free medium for keratinocytes. The NHEKs were also cultured in 0.6mM Ca for 5 days and 1.0mM Ca for 24 hrs. to cause differentiation without and with stratification, respectively. Time course adhesion experiments were performed on these cells and on each Ca concentrations and compared them with the normal Ca concentration of medium which was 0.09mM Ca.

Phase contrasts photomicrographs of these experiments show that high extracellular calcium promotes cell-cell adhesion at both 0.6mM Ca concentrations and 1.0mM Ca as shown by the closer association of the NHEKs in the colonies.

Senescent cells were identified by staining these cells in different Ca concentrations with Ki67 staining and the bar chart shows that NHEK cells did not affect the proliferation at 0.6mM Ca but 1.0mM Ca and senescent cells did affect the proliferation rate but unfortunately due to lack of time this experiment could not be repeated more than once so I could not give a confirmed hypothesis on it.

Sa-βGal activity on different Ca concentrations was performed and counter staining with Nuclear fast Red showed that some enrichment of senescent cells were seen between 10 and 30 mins but no enrichment was seen in the floating cells at these time intervals .as compared to 1.0mM Ca where there was slightly slower rate of adhesion but to prove this more experiments are necessary in the future. Nevertheless the results indicate that the rate of NHEK adhesion to FN may not be helpful in the isolation of senescent NHEKs, possibly because of the confounding influence of terminal differentiation on FN adhesion.[15]

An adhesion time course was also performed on senescent cells which were sub cultured in 0.09mM Ca and was compared with early passage control NHEK cells. Time course for senescent cells were only performed at 0, 10, 30 and 50 mins because of not having enough cells for this experiment.

However, this one result showed that senescence slowed the rate of adhesion of NHEKs to FN at the 10 min time point. As compared to young cells, this indicates that overall senescent NHEK attachment to FN is slower as compared to young cells but further studies are necessary to get to a hypothesis.

## CONCLUSIONS AND RECOMMENDATIONS

From the present study it was concluded that, NHEK differentiation alone (0.6mM Ca) did not influence the rate of NHEK adhesion to FN but differentiation accompanied by stratification (1.0mM did) and NHEK senescence did seem to reduce the rate of NHEK adhesion to FN. The reduced rate of NHEK adhesion to FN was accompanied by a reduction in the number of proliferating NHEKs.

It is recommended that such studies should be done on other cell lines as well to explore the senility and the role of senile cells with oral and maxillofacial pathologies.

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## CONTRIBUTIONS BY AUTHORS

**1 Samrina Mohammad:** Data collection & write up.

**2 Muslim Khan:** Proof reading.