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# Expression of the α5 integrin gene in corneal epithelial cells cultured on tissue-engineered human extracellular matrices

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ABSTRACT

The integrin α5β1 plays a major role in corneal wound healing by promoting epithelial cell adhesion and migration over the fibronectin matrix secreted as a cellular response to corneal damage. Expression of α5 is induced when rabbit corneal epithelial cells (RCECs) are grown in the presence of fibronectin. Here, we examined whether α5 expression is similarly altered when RCECs or human corneal epithelial cells (HCECs) are grown on a reconstructed stromal matrix used as an underlying biomaterial. Mass spectrometry and immunofluorescence analyses revealed that the biomaterial matrix produced by culturing human corneal fibroblasts with ascorbic acid (ECM/35d) contains several types of collagens, fibronectin, tenascin and proteoglycans. Results from transfection of CAT/α5-promoter plasmids, Western blot and EMSA analyses indicated that ECM/35d significantly increase expression of α5 in HCECs as a result of alteration in the expression and DNA binding of the transcription factors NFI, Sp1, AP-1 and PAX6. The biological significance of this biomaterial substitute on the expression of the α5 gene may therefore contribute to better understand the function played by the α5β1 integrin during corneal wound healing.


## 1. Introduction

Tissue engineering has led to enormous progresses in the development of technologies allowing reconstruction of autologous tissues and the development of new *in vitro* models to study both the cellular and molecular mechanisms that are critical to proper wound healing of damaged tissues. The reconstruction of corneas *in vitro* using all three corneal cell types (epithelial, stromal and endothelial cells), mostly with immortalized cell lines or animal cells, has been previously reported [1–7]. Of the corneas developed as potential tissue substitutes for transplantation, the model developed by the Laboratoire d'Organogenèse Expérimental (LOEX) uses a self-assembly approach for the reconstruction of human tissue-engineered corneas using all three native corneal cell types [8–10]. Such fully biologic corneal substitutes developed from untransformed human corneal cells without the addition of any synthetic material have been reported to show excellent corneal morphology and histological properties such as expression of epithelial keratins, integrins, basement membrane (BM) components, collagenous matrix (stroma), $Na^+/K^+$-ATPase [8–10]. These reconstructed corneal biomaterial characteristics are very close to those of human native corneas. Besides being a future treatment for many corneal disorders, such a tissue-engineered cornea also represents an outstanding biomaterial to study corneal wound healing [9].

Because of its position in the eye, the corneal epithelium is continuously exposed to several types of injuries. Damages to the corneal epithelium will rapidly activate wound healing in order to maintain a proper visual acuity. Cell–cell and cell–matrix interactions play important roles in the maintenance of the stratified structure of the corneal epithelium. Corneal wound healing is primarily regulated by growth factors, cytokines as well as components

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from the extracellular matrix (ECM) [11–14]. Integrins, a large family of transmembrane receptors that mediate inside-out signaling between the ECM and the cell, play a major role in this process [15–17]. To date, 18 α and 8 β integrin subunits that can heterodimerize into 24 integrin receptors have been reported [15,16,18,19]. Although many integrins have been identified in the corneal epithelium [20,21] (also reviewed in Ref. [13]), only a few (including integrin subunits α5, α6, α9 and β4) have been firmly documented to seek their expression altered in response to the rapid changes in the composition of the ECM occurring during the wounding process [22–24].

Damage to the cornea causes a rapid healing response that also alters the composition of the corneal epithelial basement membrane, a specialized ECM enriched in collagen type IV (CIV) and VII, laminin (LM) type-1 (LM-111), -5 (LM-332) and -10 (LM-511), entactin, and heparin sulfate proteoglycan [25–28] that separates epithelial and endothelial cells from the corneal stroma (recently reviewed in Ref. [13]). In the early steps of the corneal wound healing process, cells surrounding the wound secrete a temporary matrix that promotes epithelial cell adhesion and migration [29–31]. Both the stromal keratocytes and the basal epithelial cells contribute to the production of this temporary ECM mostly enriched with fibronectin (FN). On the other hand, collagen types I (CI) and IV (CIV) and LM temporarily disappear until the denuded area is covered, and then sequentially reappear beneath the newly reconstructed epithelium as the FN staining progressively diminishes [26,30–32]. It is now well established that the FN binding integrin α5β1 plays a major role in corneal wound healing by promoting epithelial cell adhesion and migration over the temporary and newly synthesized FN matrix [30,33].

Over the past few years, we investigated how the ECM components FN and LM may alter the expression of the integrin subunits α5 and α6 at the gene promoter level. We previously demonstrated that FN positively regulates in a cell density-dependent manner the activity directed by the human α5 and α6 integrin genes in primary cultured rabbit corneal epithelial cells (RCECs) by improving both the expression and DNA binding of transcription factors (TFs), such as Sp1 and AP-1, that are critical to transcriptional activation of these genes [34–36]. On the other hand, LM was found to suppress expression of both α5 and α6 in corneal epithelial cells by reducing the nuclear concentration of the TFs Sp1 and AP-1 and by improving the expression of members from the NFI family of TFs that act as transcriptional repressors of these genes [37,38]. Consequently, different components from the ECM appear to exert totally different regulatory influences on the expression of the α5 gene in corneal epithelial cells when taken individually. However, their combinatorial influence has yet to be determined.

In the present study, we determined the precise composition of the ECM secreted by human corneal fibroblasts following the auto-assembling model [10] and evaluated to which extent this reconstructed, complex biomaterial affects the expression of the α5 integrin subunit gene at both the protein and mRNA level in human corneal epithelial cells.

## 2. Materials and methods

All experiments described in this study were conducted in voluntary compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all procedures were approved by the Laval University Animal Care and Use Committee. This study was also conducted in accordance with our institution's guidelines and the Declaration of Helsinki. The protocols were approved by the hospital and Laval University Committees for the Protection of Human Subjects.

### 2.1. Cell culture and matrix production

Human corneal epithelial cells (HCECs) were isolated from the limbal area of normal eyes of a 52 year old donor (for transfection, Western blot or EMSA analysis) or 44-, 52- and 61 year old donors (for gene expression profiling and qPCR) following a procedure previously described [39,40]; the eyes were obtained from the Banque d'Yeux Nationale of the Centre Universitaire d'Ophtalmologie (CHU de Québec, QC, Canada). HCECs were primary cultured with a feeder layer of irradiated murine Swiss-3T3 fibroblasts (ATCC, Rockville, MD) as previously reported [40]. Rabbit corneal epithelial cells (RCECs) were obtained from the central area of freshly dissected rabbit corneas and grown into supplemented hormonal epithelial medium (SHEM) as described [38,41]. Human corneal fibroblasts were isolated from the stromal portion of a cornea (from a 26 days-old donor) left after dispase digestion and removal of both the endothelium and epithelium, and primary cultured and subcultured as previously reported [40,42]. All cells were grown under 5% $CO_2$ at 37 °C and culture medium was changed after 2–3 days.

The tissue-engineered, 3D human corneal matrix that has been used as a biomaterial on which corneal epithelial cells were cultured was produced following the self-assembly approach [9,10]. Corneal fibroblasts were seeded and cultured in fibroblast growth medium supplemented with 50 μg/ml ascorbic acid (Sigma) for either 20 or 35 days. Ascorbic acid allows fibroblasts to secrete and lay down their own ECM [9]. Fibroblasts were either left into the reconstructed matrix (living ECM; ECM/keratocytes$^+$) or removed from the 35-days ECM (devitalized ECM; ECM/keratocytes$^-$) by a deoxycholate treatment. These reconstructed biomaterial matrices were then stored at 4 °C until use.

**Table 1**
Immunofluorescence analysis of the ECM components from the corneal stroma of bioengineered and native human corneas.

| ECM component | Bioengineered cornea[a] | Native cornea |
|---|---|---|
| Collagen I | + | + |
| Collagen II | – | – |
| Collagen III | – | – |
| Collagen IV | + | + |
| Collagen V | ++ | + |
| Collagen VI | ++ | ++ |
| Collagen VII | + | + |
| Collagen XII | ++ | + |
| Collagen XIV | ++ | – |
| Fibronectin | +++ | + |
| Laminin | + | + |
| Tenascin | +++ | – |

[a] Bioengineered cornea: 2 sheets of ECM/35d on which HCECs were seeded.

### 2.2. Plasmids and oligonucleotides

The plasmid -132α5-CAT that bear the chloramphenicol acetyltransferase (CAT) reporter gene fused to a DNA fragment from the human α5 gene upstream regulatory sequence extending up to 5′ position -132 has been previously described [43,44]. The plasmid PXGH5, which bears a secreted version of the

**Table 2**
DNA sequence of the primers and double-stranded oligonucleotides.

| Oligonucleotides used as labeled probes or competitors in the EMSAs | | |
|---|---|---|
| Oligonucleotide | Top strand (5′-3′)/Bottom strand (5′-3′) | |
| **Sp1** | GATCATATCTGCGGGGCGGGGCAGACACAG<br>GATCCTGTGTCTGCCCCGCCCCGCAGATAT | |
| **NFI** | TTATTTTGGATTGAAGCCAATATGAG<br>CTCATATTGGCTTCAATCCAAAATAA | |
| **AP-1** | GATCCCCGCGTTGAGTCATTCGCCTC<br>GATCGAGGCGAATGACTCAACGCGGG | |
| **PAX6** | GATCATCCAGGTCTACTACATTAGTTCCAGGTCAG<br>GATCCTGACCTGGAACTAATGTAGTAGACCTGGAT | |
| Primers for qPCR analyses | | |
| Gene | Forward primer (5′-3′)/Reverse primer (5′-3′) | Genebank # |
| **α5** | CCATTCACAGTTCTTTGGG<br>GAGCTGCCAGGTCTTAACTC | NM_002205 |
| **MMP9** | GACGGGTATCCCTTCGAC<br>CCGAGTTGGAACCACGAC | NM_004994 |
| **MMP10** | GCTCTGCCTATCCTCTGAG<br>CACATCCTTTTCGAGGTTG | NM_002425 |
| **ACTB** | CAAGATGAGATTGGCATGG<br>GGCCACATTGTGAACTTTG | NM_001101 |