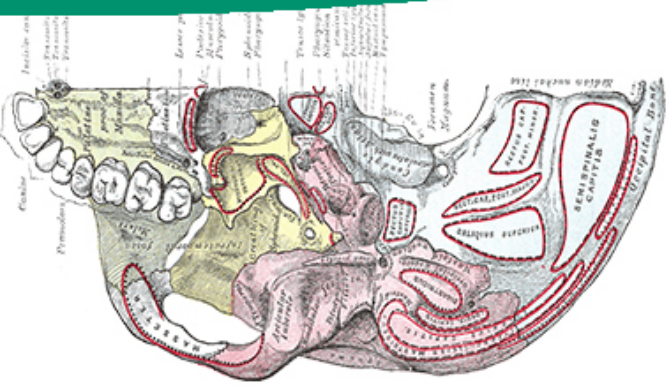

8. Original 1

Development and comparative study of basement membrane in human lining epithelium generated through tissue engineering

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Abstract:
Aim: Any complex body tissue comprised of stroma and epithelium ineluctably requires the correct development and expression of a basement membrane (BM) for its complete functionality. BMs are structures with an important role in the migration, differentiation and proliferation of cells, providing structural support and allowing a correct epithelium-stroma union and interaction. To build a complex tissue through tissue engineering the adequate development of a BM that allows the correct interaction between stroma and epithelium and therefore replicate the native tissue as accurately as possible is thus of an utmost importance. The goal of this paper was the study and contrast of BMs development in these tissues, as well as the identification of BMs components in different artificial tissue models, generated with a fibrin and agarose matrix, in order to determine whether said constructs are fit for a possible future clinical application.
Methods: oral mucosa, skin and cornea constructs were made with a fibrin and agarose (0.1%) matrix and evaluated through BM component-specific histological and immunohistochemical techniques.
Results: in every artificial tissue it is possible to observe the formation of a more or less defined BM from the third week of in vitro development, with different time patterns but sharing a common strong expression of laminin and collagen I, III and IV. Some of them even start expressing type I collagen from the first week. Conclusions: these results suggest that, from the third week, artificial tissues are able to generate properly developed and functional basement membranes, and show that lining

epitheliums created with a fibrin-agarose matrix are optimal for artificial tissue development.
Resumen:
Objetivos: Cualquier tejido corporal complejo compuesto por un estroma y un epitelio requiere necesariamente del correcto desarrollo y expresión de una membrana basal (MB) para tener funcionalidad completa. La MBs son estructuras con importantes funciones en la migración, diferenciación y proliferación de las células de dichos tejidos, sirviendo como soporte de los mismos y permitiendo una correcta unión e interacción epitelio-estromal. Para construir un tejido complejo mediante ingeniería tisular es, por tanto, de vital importancia el adecuado desarrollo de una MB que permita la correcta interacción entre el estroma y el epitelio y que, de esta manera, reproduzca el tejido nativo con la mayor fiabilidad posible. El objetivo de este trabajo fue el estudio y comparación del desarrollo de MBs en los mismos, y caracterizar los componentes de las MBs en diferentes modelos de tejidos artificiales generados con una matriz de fibrina y agarosa para determinar si dichos constructos son adecuados para una posible futura aplicación clínica. Métodos: Los constructos de mucosa oral, piel y córnea se fabricaron con una matriz de fibrina y agarosa (0,1%), y se evaluaron mediante técnicas histológicas e inmunohistoquímicas específicas para componentes de MB.
Resultados: En todos los tejidos artificiales podemos observar la generación de una MB más o menos definida a partir de la tercera semana de desarrollo in vitro, con diferentes patrones temporales pero todos con una fuerte expresión de laminina y colágenos I, III y IV. Algunos de ellos, incluso, empiezan a expresar colágeno tipo I desde la primera semana de evolución.
Conclusiones: Estos resultados sugieren que a partir de la tercera semana, los tejidos artificiales son capaces de generar membranas basales correctamente desarrolladas y funcionales, y demuestran que los epitelios de revestimiento generados con una matriz de fibrina-agarosa son óptimos para el desarrollo de tejidos artificiales.
Keywords: Basement Membrane, Tissue Engineering, Epithelium-stroma interaction, Laminin, Fibrin-agarose.
palabras clave: Membrana Basal, Ingeniería Tisular, Interacción epitelio-estromal, Laminina, Fibrina-Agarosa

Introduction

More than three decades ago, basement membranes (BMs) were described as structures of membranous nature, capable of isolating and, at the same time, connecting the cells with the surrounding connective tissue (1). Since then, it has been proved that BMs are actually specialised cellular matrices, with specific components with important roles in cell migration, differentiation and proliferation. Accordingly, the formation of a proper BM is a requirement for a correct tissue function and development (2,3).

In the same way, basement membrane's individual components are able to not only regulate different biological activities such as cell development, growth and migration, but also to detect and modulate the concentration of growth factors and cytokines in the surrounding medium (4,5). Lastly, BMs work as a basic support to determine the epithelium's polarity, and represent the fundamental structure in these cell's union with the subjacent extracellular matrix (6,7). To this amalgam of vital functions for the correct development of tissues we must add that, due to recent studies about their components and isoforms, the complexity of BM composition has been greatly increased (8).

Likewise, BM's participation in such important pathologies as Junctional Epidermolysis Bullosa, caused by a mutation of the protein Laminin 5 (or Laminin 332), which is an essential element in the BM that joints dermis and epidermis, lets us grasp the importance of this structure in the correct union between epithelium and stroma (9). Tissue Engineering has the goal of generating tissue equivalents possessing the biological, histological and functional properties of the native tissue, aiming to be an alternate solution to many pathologies. In this context, the generation of these substitutes strongly depends on the development of a basement membrane

with the aforementioned characteristics, to make the generated tissue have the necessary functions and structures to be useful in therapy.

Even though Tissue Engineering has been defined by several authors as an area of intense research activity in the last few years, with a huge potential for the repair and/or replacement of damaged tissues and organs (10, 11, 12), the missing necessary step for these tissues to become useful for the patient in a clinical setting is that they are thoroughly analysed and characterised in the laboratory, hence serving as a tool for the development of more complex and reliable tissues.

Accordingly, the goal of this work is the study of BM development in different models of artificial tissues with lining epithelium (such as oral mucosa, skin and cornea) created through Tissue Engineering techniques developed by the Tissue Engineering Group of the University of Granada (13), and to conduct a comparative study of these BMs in the different generated tissues, observing the differences in composition and in their temporal and spatial development pattern.

Furthermore, this study has the goal of characterizing the components of the BM built by the artificial tissue, with the purpose of finding out whether these constructs optimally generate the BM components observed in native tissues.

Material and methods

Artificial tissues

The artificial tissues used in the work for the study and comparison of their basement membranes come from the Histology Department of the Faculty of Medicine of the University of Granada, and were made by the Tissue Engineering group of the same department (13, 14, 15). The artificial corneas were fabricated from New Zealander albino rabbits' corneas and processed to isolate the corresponding cell populations: endothelial cells, epithelial cells and stromal corneocytes (13). Oral mucosa and artificial skin were generated from human biopsies of oral mucosa and skin, and were processed to isolate fibroblasts and keratinocytes (14, 15).
All of them were properly cultured and afterwards equivalents were built with the stromal biomaterial of fibrin and agarose (0.1%), always following the protocols designed by the Tissue Engineering Group of the Faculty of Medicine of the University of Granada (13).

Of these artificial tissues (cornea, skin and oral mucosa, respectively), "in vitro" samples were obtained at different time points : 1 week, 2 weeks and 4 weeks, with the aim of studying the progressive development of the BM in these tissues.

Also, control samples of oral mucosa, normal human skin and cornea were obtained in order to compare the basal membrane of each native tissue with artificial tissues.

Immunohistochemical Methods

All samples were fixed in 4% formaldehyde, dehydrated and then embedded in paraffin.
Four-micrometer sections were made. Next, the samples were processed. First, the samples were immersed in Xylene (3 containers, 10 minutes each) to remove paraffin, and rehydrated in graded alcohols (100% - 96% - 70% - 50% in distilled water) to be later stained.
This way, samples were immersed in Harris haematoxylin solution for 3 minutes, rinsed under tap water, and immersed in Eosin for 1 minute.
Finally, samples were immersed in graded alcohols (50% - 70% -96% - 100%) and xylene, and were properly mounted.

Histological Methods

To identify which components of the basal membrane were expressed in the different tissues, and which was their temporal expression pattern in vitro, immunohistochemical analysis of laminin, collagen I, collagen III and IV collagen was performed.
Thus, in both artificial and control paraffin-embedded samples, antibodies collagen I (ABCAM), collagen III (ABCAM ab778) and collagen IV were used against laminin 5 (Chemicon), (Master Diagnostica clone CIV22 PHM-12), at 1: 1000, 1:

200, 1: 250 and 1: 250 dilution respectively.
Briefly and in general -each antibody has its own characteristics and, therefore, there exist slight differences when standardising the technique- the process of immunohistochemical detection was as follows: xylene was used to remove all paraffin from these samples and these were rehydrated by descending alcohol series until distilled water was reached.
Next, the samples were immersed in citrate buffer 0.01 M (PH 6.0) to 90 for 25 minutes for antigen retrieval. After washing them once again in distilled water, 3% hydrogen peroxide, diluted in methanol, was used to block endogenous peroxidase activity.
The samples were washed in PBS and specific antigenic sites were blocked using casein solution to later incubate the primary antibody, diluted in casein solution. Next, samples were rewashed in PBS to incubate the secondary antibody (impress polymer reagent vector), and the samples were revealed.
Finally, samples were stained using Harris haematoxylin, dehydrated and properly mounted.

Microphotography

After haematoxylin and Eosin staining, results were photographed to facilitate the comparison between the different tissues at different times of evolution. To do so, the samples were examined in an IX81-Olympus microscope, and images were captured using a digital camera (Olympus DP-71) and the Olympus DP-BSW software. (16).

Results

Histological study of the development of artificial basement membrane tissues

The results obtained after haematoxylin and eosin staining (Figure 1) show that the three artificial tissues develop a similar temporal growth pattern: all of them show a progressive growth of the epithelium, noticeable keratinisation of the skin from the third week, and an increase in extracellular matrix cells.
The samples thus develop a growth pattern that resembles the control tissue, which can be seen optimally in the fourth week of in vitro development.
Besides, the lining epithelium and stroma remain connected in all tissues even from the first week. Eosin staining can be observed in the basement membrane from the third week, being more noticeable in the fourth week (more visible in the case of the oral mucosa).

Immunohistochemical study of the basement membrane proteins of the artificial tissues

After conducting an immunohistochemical study of laminin and collagen types I, III and IV in the basement membrane (Figure 2), the following results were obtained results:

Laminin is clearly expressed in all three tissues with the following temporal pattern: there is no expression of any kind during the first week; an intracellular expression in the epithelial cells can be observed within two weeks; laminin is clearly expressed in the basement membrane and the most basal epithelial cells from the third week.

Collagens I, III and IV, follow a different expression pattern: once again, no expression was observed during the first week, except in the case of collagen I, the only protein that appears early in the basal membrane, this being more visible in the artificial cornea.

However, during the second and third weeks (particularly in the third week) a clear intracellular staining of the three types of collagen, both in epithelial cells and in cells from the extracellular matrix can be observed, with a stronger staining on the basement membrane than in the remaining tissue.

In the fourth week, the extracellular matrix itself already contains collagens I, III and IV. It is also possible to observe an intracellular staining in all cells of the tissue (both epithelial and fibroblast, similar in all three artificial tissues) and, especially a strong signal of the basement membrane.

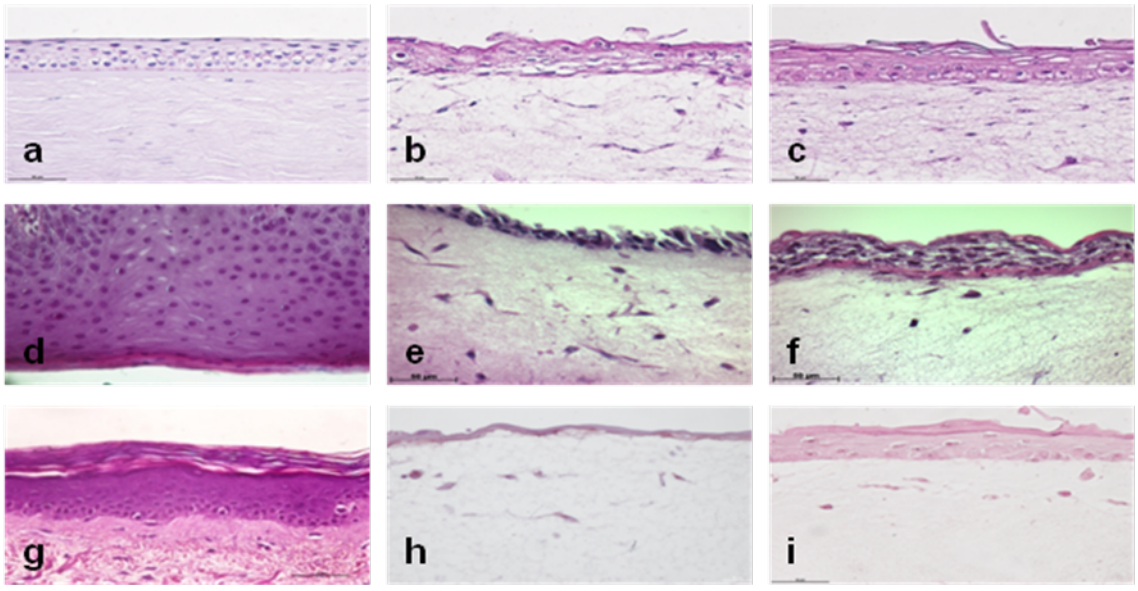

Figure 8.1: Sections stained with hematoxylin-eosin and photographed at 40x magnification. A: Human cornea control. B: 1-week in vitro artificial cornea C: 4-week in vitro artificial cornea D: Human oral mucosa control. E: 1-week in vitro artificial oral mucosa. F: 4-week in vitro artifical oral mucosa. G: Human skin control. H: 1-week in vitro artificial skin I: 4-week in vitro artificial skin

Discussion

With the results obtained from this study, its patent that artificial tissues generated off a fibrin and agarose (0,1%) matirx are capable of generating a more or less defined basament membrane. This basement membrane contains the protein elements that one could expect (laminin and collagens III and IV), along with a surprisingly strong expression of collagen I.

With the histological study, we can observe the existance of a junction between between epithelium and stroma in all the studied artificial tissues from the first week. This indicates the presence of a ”primal” basement membrane capable of binding the tissues from the first moments, preventing gaps from appearing.
The immunohistochemical study does not allow us to elucidate the exact components of these hypothetical ”primal basement membrane”. However we do observe that collagen I is one such element, being the only one marked positively in the tissues after 1 week of in vitro development.

After analyzing the rest of expressed proteins, we can point out that laminin curiously begins with an intracellular pattern observed in the epithelial level in the first few weeks. This would suggest that epithelial cells are the main origin of its synthesis. Meanwhile, collagens I, III and IV show important marking at both the epithelial and stromal levels. This could indicate that they are simultaneously generated by epithelial cells and fibroblast.

Furthermore, the only protein we were capable of observing in some tissues (cornea and skin) within the first week of in vitro development is collagen I, which could indicate either that the element is produced in larger quantities by the cells in the studied artificial tissues, helping in the formation of the basement membrane through unknown biochemical mechanisms (since collagen I is not a main component in normal human tissues) or is the one component most promptly generated. One possible way of expanding this work would consist in studying the proteins generated in the primal BM in normal human tissue, such as FAK and fibronectin.

As for the differences found among the different cell types, it was possible to observe that

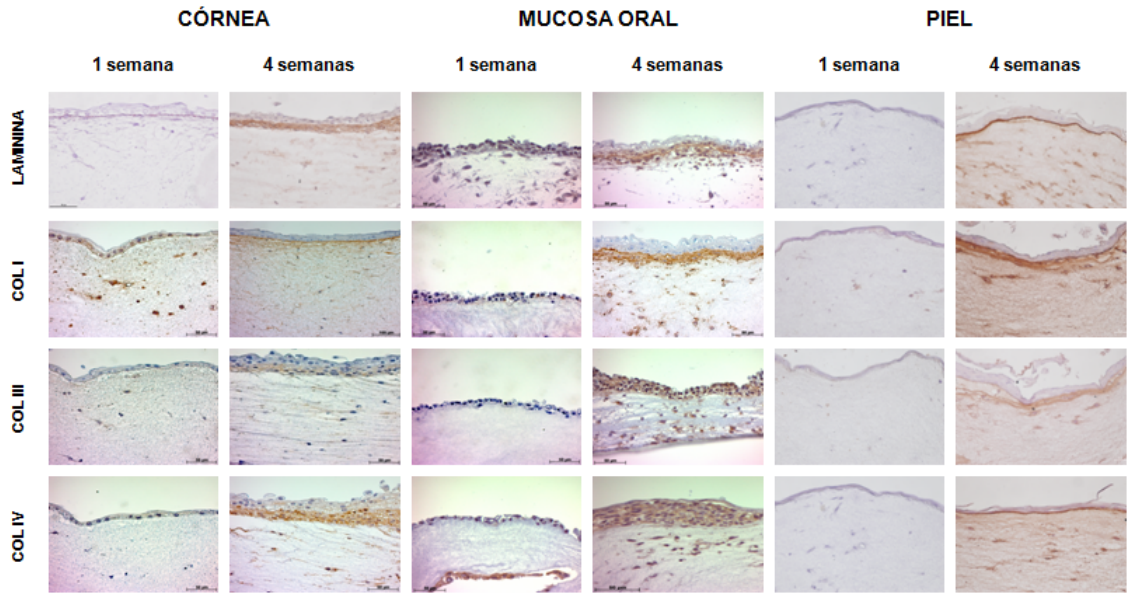

Figure 8.2: Cornea, oral mucosa and artificial skin samples after being subjected to techniques for the detection of laminin (upper row), collagen I (second row), collagen III (third row) and collagen IV (fourth row) within the constructs both 1 and 4 weeks after development.

corneal epithelial cells are capable of developing a more or less defined BM after 2 weeks of in vitro development, while skin and oral mucosa cells took one week longer in showing such a well-defined pattern. This would suggest that artificial cornea generates BM components more promptly than the rest of the epithelial-stromal tissues. However, with time the rest of the tissues end up generating such a pattern.

One possible research path would be to focus on the study of the extracellular matrix, after demonstrating in this study the capacity of these tissues of generating a well-defined BM, aiming to study if the components in the matrix are equally expressed and with the same efficacy.

If the tissues produced by the Tissular Engineering Group of the University of Granada (13) are indeed capable of generating well-defined, functional BMs with the adequate components of a native BM, and fibroblasts are capable of generating the same components of an extracellular matrix that they generate in native stroma when immerse in a fibrin and agarose (0,1%) biomaterial, it would be patent the quality of these tissues and their similarity to the microscopic pattern observed in natural human tissues. Therefore, characterizing the proteins synthesized in artificial tissues, in comparison with those that we can observe in normal tissues, constitutes a great quality control for them, in regards to the optimization of them for future possible therapeutic uses.

Conclusion

After analyzing the results and comparative study of the temporal expression pattern of the BM in the generated tissues by techniques of tissular engineering, we can conclude that:

1. Artificial tissues generated with a fibrin-agarose (0,1%) matrix following the protocols established by the Tissular Engineering Group of the University of Granada, are capable of developing a well-defined BM with its principal protein components.
2. Said BM in these surface epithelia develops optimally after 3 weeks of in vitro development, albeit the corneal cells generate a well-defined membrane from the second week on.
3. Laminin is primarily synthesized by the epithelial cells, while the rest of the com-

ponents are synthesized between epithelial cells and fibroblasts from the extracellular matrix.

4. Collagen I plays an important role in generating the BMs in artificial tissues, being the only protein expressed promptly from the first week and offering an intense signal in the BM and the lower layers of the epithelium.
5. Generally speaking, the studied artificial tissues are capable of maintaining the epithelium-stroma interaction in a similar fashion as the one found in human tissues, which makes it a great tool in quality control of any tissue generated by tissular engineering and represents promise of possible future clinical use of these tissues as a therapeutic option.

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