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Original Article

Evaluation of lymphangiogenesis in acellular dermal matrix

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ABSTRACT

Introduction: Much attention has been directed towards understanding the phenomena of angiogenesis and lymphangiogenesis in wound healing. Thanks to the manifold dermal substitute available nowadays, wound treatment has improved greatly. Many studies have been published about angiogenesis and cell invasion in INTEGRA®. On the other hand, the development of the lymphatic network in acellular dermal matrix (ADM) is a more obscure matter. In this article, we aim to characterize the different phases of host cell invasion in ADM. Special attention was given to lymphangiogenic aspects. **Materials and Methods:** Among 57 rats selected to analyse the role of ADM in lymphangiogenesis, we created four groups. We performed an excision procedure on both thighs of these rats: On the left one we did not perform any action except repairing the borders of the wound; while on the right one we used INTEGRA® implant. The excision biopsy was performed at four different times: First group after 7 days, second after 14 days, third after 21 days and fourth after 28 days. For our microscopic evaluation, we used the classical staining technique of haematoxylin and eosin and a semi-quantitative method in order to evaluate cellularity counts. To assess angiogenesis and lymphangiogenesis development we employed PROX-1 Ab and CD31/PECAM for immunohistochemical analysis. **Results:** We found remarkable wound contraction in defects that healed by secondary intention while minor wound contraction was observed in defects treated with ADM. At day 7, optical microscopy revealed a more plentiful cellularity in the granulation tissue compared with the dermal regeneration matrix. The immunohistochemical process highlighted vascular and lymphatic cells in both groups. After 14 days a high grade of fibrosis was noticeable in the non-treated group. At day 21, both lymphatic and vascular endothelial cells were better developed in the group with a dermal matrix application. At day 28, lymphatic endothelial cells had organized themselves, engineering the pseudocylindrical structure better disposed in the ADM group than in the control group, and the lymphatic cells were detectable inside the vessels' lumen in this group. **Conclusion:** This study has made it possible to demonstrate the absolute importance of an ADM in proper wound healing and has shown better definition of both the qualitative and quantitative aspects of lymphangiogenesis compared to the second intention healing. A major grade of organization of the extracellular matrix and a minor grade of fibrosclerosis in ADM allowed a well-structured morphologic and functional development of the endothelial and lymphatic vascular structures. This study hopes to represent a clinical basis for a wider use of ADM in lesions where lymphatic complications are common.

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KEY WORDS

Acellular dermal matrix; INTEGRA®; lymphangiogenesis

INTRODUCTION

Acellular dermal matrices (ADMs) such as INTEGRA® are usually employed for abdominal, breast,[1-4] head[5,6] and neck reconstruction, genital[7] and in post burn injuries,[4,8,9] ulcers[10], cancer[5,11,12] and post trauma[13] surgery. The clinical importance of these skin substitutes derives from the fact that they are resistant and pliable and can be rapidly integrated into the surrounding tissues.[14-18] The biological benefits of INTEGRA®; INTEGRA® are manifold. Their incorporation in tissue is thought to improve revascularization of the matrix, invasion of adjacent host-derived cells and their transition into specific tissue based on the local microenvironment.[14]

Despite the fact that others have studied[19,20] the histological aspects of INTEGRA® explants in various settings and the characterization of host cell invasion into ADM, the development of the lymphatic network has not been described in the literature. A broader understanding of lymphangiogenesis into INTEGRA® may better define the current use in reconstructive surgery.[16,17,21] Given the potential usefulness of ADM, we wish to characterize lymphatic invasion into the matrix at defined time intervals. We have used a model in which INTEGRA® was implanted in the lateral rat thighs. The tissue was harvested at 7, 14, 21 and 28 days for subsequent histologic analysis. We analysed the wound contraction in ADM compared with secondary intention wound healing and characterized host cell invasion using cell type specific immunohistochemistry: Anti-PECAM for vascular angiogenetic characterization and anti-PROX1 for lymphangiogenetic definement.

Based on observations made from our initial histological analysis, we hypothesized that INTEGRA® is capable of supporting lymphangiogenesis.

MATERIALS AND METHODS

After the consent for the execution of this study by Local and National Animal Ethical Committee we selected 57 Sprague-Dawley murine models. We removed two 1.5 cm^2 full thickness tissue lozenges on the lateral side of both rat thighs. As previously described in other studies,[22,23] we opted for thigh model in order to have symmetric results and to easily show the changes during the period of evaluation. The left thigh defect healed by second intention. The right one was covered by INTEGRA®, an ADM comprised of cross-linked bovine tendon collagen and glycosaminoglycan (GAG) broadly used as skin replacement in chronic and traumatic, pressure, diabetic, chronic wounds, and vascular ulcers.[12,16,24-29]

Among the 60 rats, we formed four excision biopsy groups so divided:

- 15 rats: Biopsy after 7 days
- 15 rats: Biopsy after 14 days
- 15 rats: Biopsy after 21 days
- 15 rats: Biopsy after 28 days.

After general anaesthesia with (ketamine cloridate [80-100 mg/kg] + Xilazine cloridrate [10 mg/kg]), bilateral trichotomy of lateral side of the thigh, disinfection with clorhexidine 0.5%, we proceeded with a bilateral incision until the fascial plane; we made curettage of the fascial plane until obtaining only a light and homogeneous bleeding. Accordingly, an artificial dermal matrix sheet of 1.5 cm was laid down on the right side and fixed with metallic clips. Both wounds were treated with non-adherent dressing and Elizabethan collar was positioned. During the post-operative period, we checked the wounds, and we cleansed the areas with saline every 72 h.

We performed excisional biopsy, after chlorhexidine 0.5% disinfection, and we took off the metallic clips and the silicon layer; a skin and subcutaneous tissue incision with 2 mm of healthy tissue was performed by removing a square of tissue including neodermis on the right-hand side and granulation tissue on the left. At the end of the procedure, the laboratory animals were killed by intravenous injection of the lethal dose of KCl.

In order to attest INTEGRA®'s capability of supporting lymphangiogenesis, we have performed histological staining with haematoxylin and eosine and immunohistochemical analysis with PROX-1 Ab[30-35] for endothelial lymphatic cells and CD31/PECAM[36-38] for endothelial hematic cells.

In order to evaluate the amount of cellularity in the tissue implanted with ADM and a semi-quantitative analysis was used.

To maintain an objective evaluation, and to measure wound areas and contraction, we completed a semi-quantitative analysis at days 7, 14, 21 and 28.

RESULTS

Macroscopically a considerable contraction in wounds healed by second intention (30% at day 7 and 50% at day 14; 60% at day 21 and 80% at day 28) was observed; however the contraction of defects covered with dermal matrix (approximately 5% at day 7, 7% at day 14; 10% at day 14, 13% at day 28) was more limited Graphic 1 and Figure 1.

Microscopic results and lymphangiogenesis

Results at 7 days

Optical microscopy showed a more plentiful cellularity in granulation tissue compared to the regeneration dermal matrix as demonstrated with a semi-quantitative cell counting of 100× zoom images. We divided the sections into different zones, and we discovered a higher quantity of cells in or in the area of the muscular fascia to the superficial skin. Examination of the tissue sections at 7 and 14 days post-implantation revealed a directly proportional correlation of the cellular density with implantation time. Our semi-quantitative analysis of average cell quantity showed a 4-fold increase in granulation tissue at 14 days. Also, within the INTEGRA® there was a 1.5-fold increase at 14 days. It had been invaded by phagocytes (neutrophils and macrophages), which had immigrated through the three-dimensional scaffold. The cellular component followed a gradient rising from the wound's bed to matrix the surface.

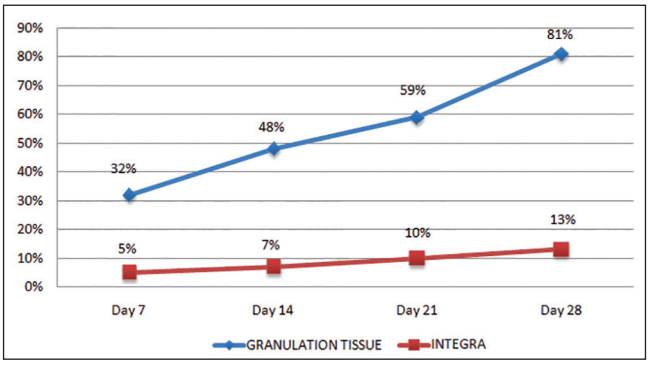
This immunohistochemical process [Figures 2a, b and 3] highlighted a vascular endothelial cellular proliferation (PECAM +) located, above all, at the borders and at the bed of the lesion and uncommon lymphatic vascular cells (PROX-1 +) both in the granulation tissue and dermal matrix (INTEGRA®) [Figures 4-6].

Results at 14 days

Fourteen days after implantation, it was possible to observe the degradation of dermal matrix collagen by phagocytes. The granulation tissue showed a notable fibrosis. Confluent endothelial lymphatic cells (PROX-1 +) were visible both in granulation tissue and dermal matrix [Figures 7 and 8].

Results at 21 days

The number of vascular (PECAM +) and lymphatic (PROX-1 +) endothelial cells grew in both tissues but in INTEGRA® dermal matrix, they formed better structured lymphatic and vascular outlines; in granulation tissue there was an evident and remarkable fibrosclerotic reaction. Semi-quantitative analysis of PROX-1 + cells



Graphic 1: Graphic resuming the difference in contraction between INTEGRA (approximately 5% at day 7, 7% at day 14; 10% at day 14, 13% at day 28) and control group (32% at day 7 and 48% at day 14; 59% at day 21 and 81% at day 28)

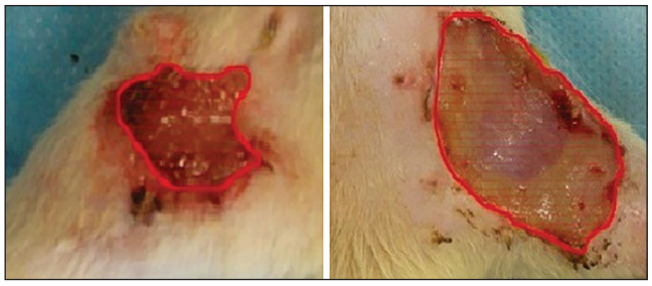


Figure 1: Left side: Macroscopic check in second intention healing of thigh wound at day 28. Notice the remarkable contraction of the granulation tissue and the initial reepithelisation. Right side: Macroscopic check in defect after application of artificial dermal matrix (INTEGRA®) at day 28. Notice the lack of wound contraction and the more acceptable appearance of the wound

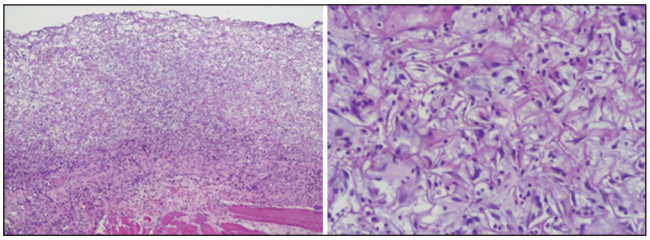


Figure 2: Microscopic results with ×200 (left) immunohistochelical process with PECAM and ×1100 (right) with PROX-1 in INTEGRA® dermal matrix at day 21. Notice, marked with red circle, a vascular outline formed by lymphatic vascular cells

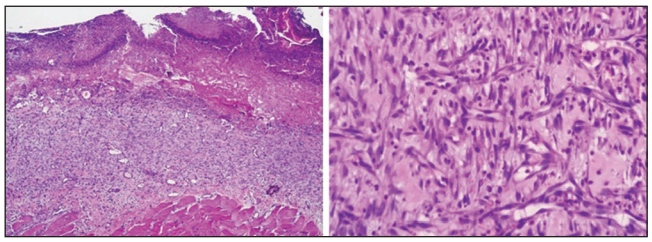


Figure 3: Microscopic results in ×200 (left) immunohistochelical process with PECAM and ×1100 (right) with PROX-1 in non-treated tissue at day 21. Notice the lower plenty and loss of organization of lymphatic vascular cells and the abundant fibrosclerosis of tissue

suggested a quantitative improvement in lymphatic cells in INTEGRA® implanted group [Figures 2 and 3].

Results at 28 days

Blood and lymphatic vessels appeared better structured and organised in the regeneration dermal matrix than the vessels in granulation tissue. Lymphatic vascular structures (PROX-1 +) with lymphocytes were detectable inside the lumen of vessels [Figures 9 and 10].

DISCUSSION

The biointegration of an ADM follows different phases, similar to the phases we find in normal wound healing: An inflammatory phase, fibroblast migration, neovascularization, remodelling and maturation.[39,40] In the inflammatory phase — the matrix is invaded by phagocytes that migrate across the matrix; in the phase of fibroblast migration — macrophages, leucocytes and fibroblasts migrate from wound borders through collagen and GAG network and dispose themselves in the three-dimensional scaffold; the neovascularization phase — demonstrable with anti-PECAM antibodies that show neoangiogenesis with a well-structured organization on hematic vessels. In the remodelling and maturation phase is observable completion of the matrix degradation and a neosynthesis of collagen by the fibroblasts. Neovascularization in INTEGRA® has been proved by several studies;[41-43] in this study we aim to prove that an ADM as INTEGRA® is capable of supporting

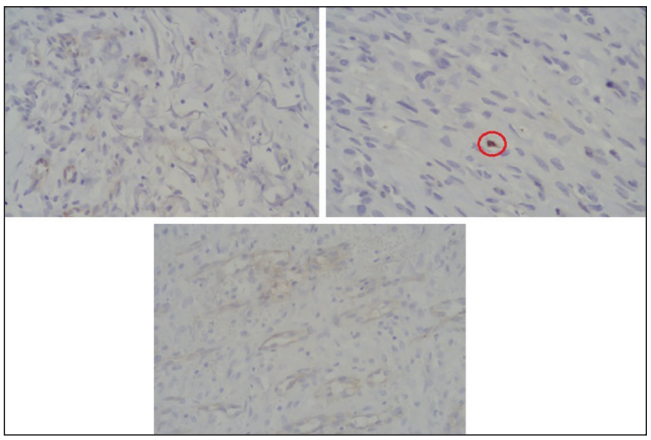


Figure 4: ×40 (left) and ×100 (right) H and E staining in tissue treated with artificial dermal matrix at day 7. Notice the massive infiltration of neutrophils and macrophages in characterization of inflammatory phase

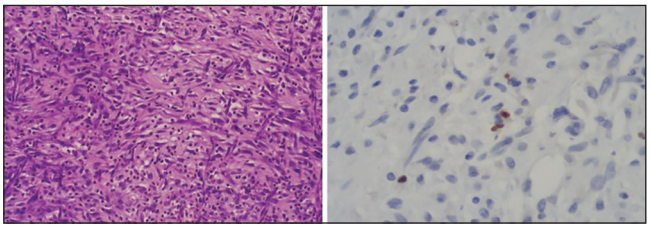


Figure 6: ×100 (left and right) H and E, ×1100 (centre) immunohistochemical process with PECAM and PROX-1 at day 7. Notice (remarked with a red circle) an endothelial lymphatic cell. In Figure 7a and b, that depict artificial dermal matrix, more vascular endothelial cells are detectable

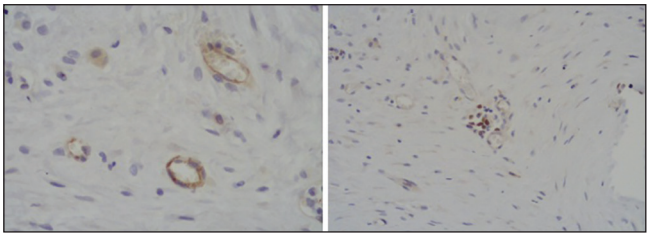


Figure 8: ×100 (left) and H and E, ×1100 (right) immunohistochemical process with PROX-1 in granulation tissue at day 14. Notice the scarce number of PROX-1 + cells in regard to Figure 5 and an initial fibrosis of the tissue

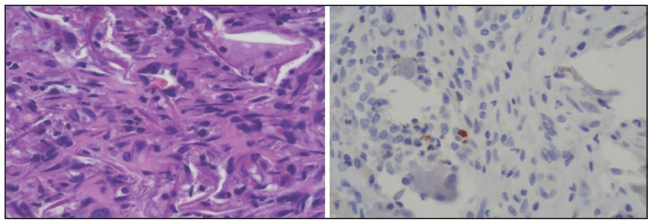


Figure 5: ×40 (left) and ×100 (right) H and E staining in granulation tissue at day 7. Notice the massive infiltration of neutrophils and macrophages in characterization of inflammatory phase and the initial reepithelisation

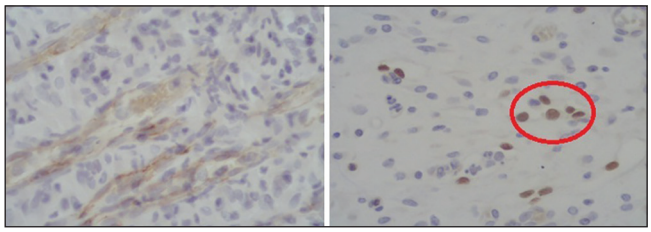


Figure 7: ×200 (left) H and E, ×1100 (right) immunohistochemical process with PROX-1 in tissue treated with artificial dermal matrix at day 14. Notice the abundant number of PROX-1 + cells

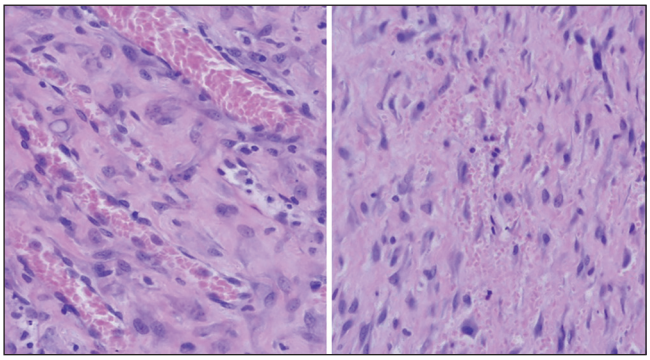


Figure 9: Comparison between artificial dermal matrix (left) and granulation tissue (right) in ×200 microscopic results with H and E staining. Notice the superiority of dermal matrix case in organisation, number and structure of blood and lymphatic vessels

lymphangiogenesis. Only few studies try to connect wound healing and lymphangiogenesis issues.[44-46] Low lymphatic vessels presence seems to be an important factor in the impairment of diabetic ulcers wound healing.[47] Moreover, lymphatic vessels are responsible for the maintenance cells equilibrium and normal wound healing. The main role of the lymphatic vessels is the control of the interstitial microcirculation. The lymphatic vessels remove macromolecules and particulate matter too large to reenter the blood capillaries from the extra vascular space. . If these materials are not removed, the osmotic and hydrostatic forces within the tissues change and disease results. Failure of the lymphatic function leads to pollution of the tissues because of the excesses of protein, other macromolecules, and fluid around the cells, resulting in impaired wound healing.[48]

This study make an effort to demonstrate the role of the artificial dermal matrix (INTEGRA®) in the proper wound healing[49,50] and underline the better structured pattern of lymphangiogenesis between the two groups considered.[22] From the 1st week of observation, a minor wound contraction in tissue treated with INTEGRA® implant was evident. A morphological examination of tissue specimen revealed that the response to the ADM was similar during wound healing, involving mostly inflammatory cells in the 1st day, and afterwards, in the mesenchymal cells. The quantitative analysis indicated that the total number of cells within the ADM increased time following a gradient rising from the wound bed to matrix the surface. Our microscopic investigation emphasized the low fibrosclerosis in ADM compared with the granulation tissue in which we observed a copious fibrosis. We found a major grade of organisation of extracellular matrix and a minor presence of fibrosclerosis in INTEGRA®; furthermore, we observed histological evidence of lymphatic vessels formation within ADM and confirmed this finding by using immunohistochemistry for the lymphatic endothelial cell marker PROX-1. Lymphatic endothelial cells initially diffused inside the thickness of dermal matrix and organized themselves constructing a pseudocylindrical structure better organized both qualitatively and quantitatively compared to granulation tissue. A well-structured morphological and functional development in vascular and lymphatic endothelial structures was observed [Figure 11]. This could give an explication about the low morbidity of the integrate site for seroma formation reported in our experiences as well in the literature. A more structured lymphatic vessels formation could be significant for a more functional lymphatic activity in the new tissue. However, our observation is not enough to understand the functional, so clinical studies still be needed to test the functionality of lymphatic channelling within the ADM.

CONCLUSION

This study made possible the demonstration of the absolute importance of an ADM in proper wound healing[55] and in the better characterization of the qualitative and quantitative aspects of lymphangiogenesis compared to healing by secondary intention. By using this simple animal model, it was possible to prove that INTEGRA supports the formation of lymphatic structures. Our

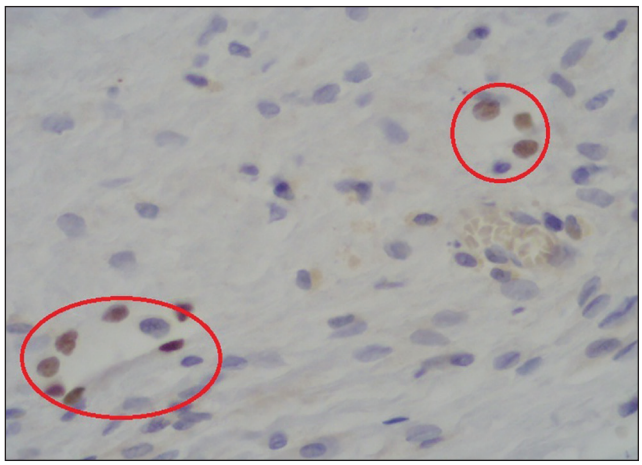


Figure 10: Lymphatic vascular structure with lymphocytes inside vessels lumen highlight by ×1400 immunohistochemical process with PROX-1 at day 28 in tissue treated with INTEGRA®

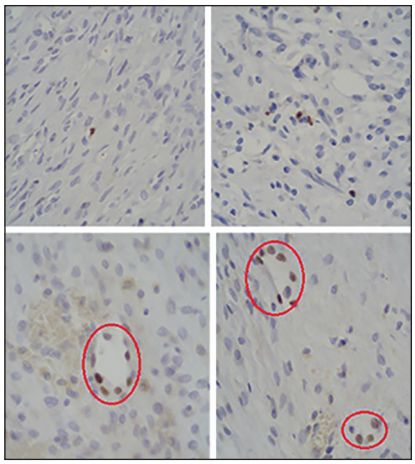


Figure 11: Immunohistochemical process with PROX-1 on tissue treated with dermal matrix (INTEGRA®) underlines an increase of amount of lymphatic endothelial cells and the formation of well-organized cylindric vascular structure (red circles in right-centre and right image). Left (day 7), left-centre (day 14), right-centre (day 21), right (day 28)

observations outline a rational theoretical basis toward which a broader clinical use of skin substitutes in lesions when the risk of lymphedema would compromise both morphological and functional outcomes.[51-54] Although further studies are necessary in order to better evaluate the development of the lymphatic network in more evolved models, such as porcine or human models, this study represents a rational start in our understanding of the reasons why ADMs have so many useful applications in plastic and reconstructive surgery.

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