Abstract. Historically, in severe trauma, such as extensive burns and large loss of substance of the soft tissues, increased emphasis is given to the survival of the traumatized person and less attention is placed on the recovery of the actual traumatized organ. Today, recovery of complete functionality of the injured part and the possibility of minimizing scars in order to make them as acceptable and invisible as possible are also important. The present study analyzed morphologically the events occurring in the wounds of patients in which a dermal substitute was used in combination with a cleansing process. Pre-treatment of the lesion by cleansing followed by the application of a biomaterial showed that in the tissue that forms, a reduced number of cells are present, the collagen is more undulating with interstitial spaces, and Langerhans cells are evident. In addition, these cells participate in the growth and turnover of keratinocytes. The mediating role of these elements would also be strongly dependent on the components of the extracellular matrix of the dermis.

The formation of new blood vessels is carried out by stimulatory and inhibitory endogenous molecules that regulate the stages of wound healing (3). When this complex process terminates, the levels of growth factors decrease, inflammation disappears, the matrix stabilizes, and endogenous inhibitors of angiogenesis are activated (4).

Historically, in severe trauma (such as extensive burns and large loss of substance of the soft tissues) increased emphasis is given to the survival of the traumatized person and less attention is placed on the actual recovery of the traumatized organ.

Nowadays, the recovery of complete functionality of the injured part and the possibility of minimizing the remaining scars to make them as acceptable and invisible as possible are also important. This has encouraged the development of research on skin substitutes (5, 6).

In our experience, skin grafting on wounds characterized by an irregular wound bed has rarely been successful (7). In fact, under these conditions, the lack of growth factors and, vice versa, an abundance of their inhibitors, slows-down wound healing. Improvement has been achieved with the use of a dermal substitute that reduces inflammation and protects from possible contamination, allowing for regeneration of neodermic tissue.

The present study analyzed morphologically the events occurring in the wounds of patients in which a dermal substitute was used in combination with a cleansing process.

Materials and Methods

Patient enrollment. The patients considered eligible subjects for the study were those with chronic ulcers of the lower extremities with flebo-lymphostatic injuries, followed-up at the Division of Geriatric and General Surgery of the Second University of Naples.

Sixteen patients were enrolled (eight males and eight females), with an age between 35 and 50 years. Half of the patients were
suffering from disorders of carbohydrate metabolism, the other half of obesity. The wounds had a diameter between 10 and 15 cm and they were all localized in the lower limb just above the malleolar region. The lesions were characterized by a great loss of tissue and subsequent externalization of the muscular fascia but not that of the tendon. All wounds had infectious processes supported by Pseudomonas or Staphylococcus aureus.

The patients were divided into two groups: only one group of patients was subject to a process in which a dermal substitute was used in combination with a cleansing process.

Only the group treated exhibited the morphological events described here. The most significant figure obtained was used to demonstrate the morphological events occurring in the wounds.

Treatment by cleansing. The patients were subjected to a process of cleansing which consisted of two phases: Step 1: We carried out TIME (Tissue, Infection or Inflammation, Moisture imbalance, Epidermal margin), which were procedural sequences whose purpose was the removal of necrotic tissue (T), the control of exudate (I), the maceration (further control of exudate) (M) and the restoration of the dermal matrix (E). Step 2: surgical cleansing was practiced through hydroscalpel (Versaget) (Smith & Nephew, Milan, Italy) to cleanse the wound. Moreover the hydroscalpel (Versaget) was used to delineate the wound margins so that the subsequent application of a biological membrane was easier and more effective. After cleansing, Integra® (Integra Biosciences, Chur, Switzerland) was applied. Integra® is a semi-biological plane membrane with histo-inductive and histoconductive action on mesenchyme, which guides the formation of a dermis similar to normal (8-12).

At the expiry of the third week, the silicone of the membrane was removed, and then a graft of skin taken from the thigh of the patient themselves was performed.

Morphological analysis. Biopsies were taken at 7, 14, 21 days after implantation of the skin. The biopsies were fixed in a solution of 0.2 M to 2.5% glutaraldehyde in phosphate buffer (PBS) for at least 2 hours and then were fixed in 1% OsO4 in PBS for 1 hour. After repeated washing in PBS, the samples were dehydrated through an ascending series of alcohols and finally embedded in Epon® 812 (Electron Microscopy Sciences, Hatfield, USA). Ultrathin sections (thickness of about 40 to 60 nm) were produced by Leica UCT ultramicrotome and placed on grids of 200 mesh (a measurement scale for small particles) on single samples. The sections were then counterstained with 4% uranyl acetate in PBS for 10 minutes and with 3% lead citrate in PBS for more 5 minutes. The grids were observed under a LEO 912 AB transmission electron microscope (TEM) (Carl Zeiss spa Milan, Italy) (13).

Results

In samples taken at day 7, there were no clear signs of inflammation; the cell number was scarce, and there were no neutrophils, plasma cells, eosinophils, lymphocytes or macrophage monocytes. We observed small cells which exhibited no defense response; these elements were progenitor cells which tended to stretch and flatten (Figure 1a and b). These cells were bound to the matrix and were surrounded by fibrillar collagen (Figure 1c). There were no vascular connections, but we observed progenitor cells and syncytial fibroblasts.

In samples taken at day 14, we observed the formation of new blood vessels (Figure 2a). The cell density increased with the formation of new cells, deriving from underlying blood vessels or mitosis of cells with the characteristics of embryonic syncytial cells. Furthermore, around the blood capillaries, it was possible to distinguish the pericytes. (Figure 2b and c). The collagen in this phase was thick and organized in a lamellar architecture, trapping fibroblast-like cells (Figure 2d).

In samples taken at day 21, we observed a uniformly established matrix, and giant cells, as well as tissue areas in which we highlighted structures which were strongly osmiophilic with organization of the desmosomal junctions. Another element that appeared was the Langerhans cells, dendritic cells that notoriously show the ability in the capture, uptake and processing of antigens Figure 3a). Moreover, an increased number of pericyte was present, associated with the production of new collagen (Figure 3b).

Discussion

The present study morphologically analyzed the events occurring in the wounds of patients in which a dermal substitute was used in combination with a cleansing process. In biopsies taken at day 7, we observed no inflammation and a poor number of cells. There were neither neutrophils nor plasma cells, eosinophils, or macrophage monocytes. However, we did observe small cells without a defense response. These cells are progenitor cells which tend to stretch and flatten out, becoming transition cells, which recognize and bind to the matrix. The link to the matrix makes sure that these cells exhibit similarities to the cells in embryonic dermatogenesis and not to those of normal postnatal life. In fact, in our preparations, as in the mechanisms of embryonic development, there were isolated clusters of cells beginning to produce fibril collagen.

In this phase, the tissue being formed had no vascular connections; cells uniformly-scattered were progenitor cells and syncytial fibroblasts. Fibroblasts, like all embryonic cells and other proliferative cells, started to produce angiogenic factors which attracted new blood vessels from surrounding areas (1).

In biopsies taken at day 14, we observed several newly-formed vessels that represented a reserve of mesodermal proliferative cells taking part in the regeneration of new skin tissue (14, 15). Therefore at this stage, migratory elongated cells invaded the matrix, starting the formation of additional vessels. These cells mostly comprised of angioblasts re-organizing new blood vessels. While the cell clusters filled the empty space with the production of fibril collagen, the collagen, in this phase, was thick and organized in a lamellar...
architecture that trapped fibroblast-like cells. Meanwhile, the
cell clusters exerted their effects on the underlying blood
vessels, which reacted with the invasion of new blood
vessels. The processes described above occurred
simultaneously at different points of the lesion and gradually
tended to converge.

We should point out that from day 14, it was possible to
observe pericytes surrounding the capillaries, and later, closer
to the capillaries (day 21). The presence of pericytes and
smooth muscle cells established intimate cell-to-cell contacts
that help coordinate vascular tonus. Pericytes are increasingly
understood to play a key role in the microvascular physiology
of the microvascular remodelling, maturation and stabilization
of angiogenesis and also lymphangiogenesis.

In biopsies taken at day 21, we observed the Langerhans
cell, a dendritic cell which normally shows the ability in the

Figure 1. Biopsies taken at 7 days after implantation of the skin. It is possible to observe that the cells are scarce, bound to the matrix and were surrounded by fibrillar collagen.

Figure 2. Biopsies taken at 14 days after implantation of the skin. It is possible to observe that the cell density increased with the formation of new blood vessels and pericytes.
capture, uptake and processing of antigens (16). Langerhans cells appeared as very clear cells by TEM. In some cases, they appeared like a rod, with a fluffy linear coating along the inner surface of a tri-laminar limiting membrane. These cells belong to the large group of dendritic cells, mononuclear phagocytes, which are important in immune reactions. From a phenotypic aspect, they possess receptors for the Fc portion of IgG and for the C3b and C4d components of complement, and express a variety of antigens, the number of which is progressively increasing: the antigens of class I and II of the major histocompatibility complex (MHC) and CD1a antigen; their cytoplasm also expresses S100 protein (17).

The Langerhans cell is the key element of the lymphoid tissue associated with skin, which is the peripheral outpost of the immunosurveillance system and is implicated in the genesis and regulation of the primary immune response. The Langerhans cell is also a determining factor in the regulation of growth and turnover of keratinocytes. While being located in the deep layers of the epidermis, the Langerhans cell is able to determine cell movements toward the surface. It is also able to move and act if necessary on the side of the dermo-epidermal junction for its dendritic shape.

In summary, this method of pre-treatment of the lesion with a process of cleansing followed by the application of a bio-material showed that in the organization of the tissue that has formed, a reduced number of cells is present, the collagen is more undulating with interstitial spaces, and Langerhans cells are evident. The Langerhans cells is a key element in the skin-associated lymphoid tissue and participate in the growth and turnover of keratinocytes. The mediating role of these elements would also be strongly dependent on the components of the extracellular matrix of the dermis.

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