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Short Communication

Apoptosis, Necrosis, and Proliferation

Possible Implications in the Etiology of Keloids

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Keloids are collagenous lesions acquired as a result of abnormal wound healing. In this study we have assessed the potential role of proliferation, apoptosis, and necrosis in keloids. Samples were immunolabeled for proliferating cell nuclear antigen or DNA strand breaks or stained with acridine orange. Proliferating cells were observed in the basal layer of the epidermis and fibroblasts in the dermis, the numbers of the latter being increased in comparison with normal skin. No proliferating cells were observed in the central region of the keloid. In normal skin, apoptotic cells were restricted to the basal layer of the epidermis. In keloid samples, numerous apoptotic cells were observed in the epidermis and dermis; the number and distribution of positive cells decreased more distal to the keloid lesion. Apoptotic endothelial cells of a small proportion of blood vessels in the dermis were also observed. Evidence of necrosis was also seen in the dermis. These results suggest that, with maturity, progressive cell degeneration primarily by apoptosis results in clearance of certain cellular populations resulting in the typical keloid lesion. However, the persistence of fibroblast proliferation at the dermal/keloid interface propagates the fibrosis. (Am J Pathol 1996, 149:1441–1447)

Keloids are collagenous, cutaneous lesions that can form anywhere on the body, usually as a result of trauma. Unlike hypertrophic scars, keloids have a tendency to outgrow the original boundary of the insult. Furthermore, they do not spontaneously regress and have thus been classified as being among the benign dermal tumors. It has been estimated that up to 16% of Blacks and Hispanics suffer from keloids.[1] At present, the majority of treatment is surgical excision. However, this frequently results in recurrence of the lesion and in many cases exacerbates the condition.[2]

During the course of normal wound healing, new tissue or granulation tissue is formed. It contains numerous tortuous capillaries and extensive fibroblast populations embedded in a collagenous extracellular matrix. Eventually, the granulation tissue is replaced by an acellular collagenous scar. During the early stages of keloid formation, excess numbers of fibroblasts and microvessels are similarly observed. This raises the question of where the new cells come from and what ultimately happens to them to result in the formation of keloids.

The balance between cell proliferation and cell death is central to the etiology of most pathologies. Apoptosis is a process by which cells die in a controlled manner.[3] It is induced by specific stimuli, the components of the cell become condensed, and it is often followed by phagocytosis. This is in contrast to necrosis, during which the cell membrane is breached resulting in release of potentially harmful cellular constituents. In view of the large numbers of the population affected by keloids, an understanding

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of the processes involved in their formation and propagation is essential. In this study, we have therefore investigated the possible role of proliferation, necrosis, and apoptosis in keloids.

Materials and Methods

Keloid samples (14 Black and 1 Caucasian) were obtained from patients undergoing surgical excision and were used immediately. For studies using cryostat sections, keloids were snap-frozen in n-Hexane in a bath of liquid nitrogen and maintained at −70°C until use. For studies using paraffin sections, samples were placed in formaldehyde and dehydrated in ascending concentrations of alcohol before wax embedding. Normal human skin served as a control.

Immunolocalization of Proliferating Cell Nuclear Antigen (PCNA)

Paraffin sections (5 μm) were rehydrated in descending concentrations of alcohols and placed in phosphate-buffered saline (PBS) solution for 5 minutes. Endogenous peroxidases were quenched with 0.3% H_2O_2 in methanol, and sections were washed in PBS. Nonspecific binding of IgGs was blocked using normal horse serum 1:50 in 0.1% bovine serum albumin (globulin free) in PBS. The sections were incubated with primary antibody (monoclonal anti-human PCNA; Dako, High Wycombe, UK) at a dilution of 1:500 for 1 hour, washed, and incubated for an additional 30 minutes with a biotinylated anti-mouse IgG secondary antibody (Vector Laboratories, Peterborough, UK). After an additional 30-minute incubation with Vectastain ABC horseradish peroxidase, the substrate (0.05% 3,3-diaminobenzidine tetrahydrochloride in Tris buffer; Sigma, Poole, UK) was added for the appropriate time period (5 to 10 minutes). This resulted in PCNA-positive cells being labeled as brown. Morphological observations were confirmed using routine histological stains, hematoxylin and eosin, and toluidine blue.

In Situ *End Labeling*

DNA strand breaks were detected according to the method of Ansari et al,[4] using the Apoptag *in situ* apoptosis kit (Oncor, Gaithersburg, MD).

Acridine Orange Staining for Nucleic Acids

Cryostat sections (10 μm) were cut and immediately stained in 1 part 0.1% acridine orange solution, 9 parts Krebs solution (pH 6.2) for 15 minutes. Sections were then blotted and mounted in Krebs solution. This resulted in DNA staining green, RNA red, and fibrous tissue yellow/green.

Results

Normal Histology

The keloid was found to be a highly organized acellular collagenous lesion. The collagen fibers were aligned parallel to the skin forming a nodule, with the longest axis running coincident to the dermis. The fibroblasts within the lesion showed a similar orientation. The center of the nodule was avascular, which was in contrast to the area of dermis juxtaposed to the keloid lesion, which contained numerous blood vessels. Within this region, numerous focal groupings of lymphocytes were observed (as determined by labeling with the pan-T cell marker CD3, data not shown), typically associated with hair follicles. Mast cells were present throughout the dermis in association with the lymphocyte populations, but few mast cells were observed in the lesion. Small numbers of macrophages, eosinophils, and polymorphonuclear neutrophils were sparsely located in the dermis.

PCNA Labeling

In normal and keloid epidermis, numerous proliferating cells in the basal layer were seen (see Figure 1a). In normal dermis, virtually no proliferation was observed. In contrast, the dermal region adjacent to the keloid contained PCNA-positive fibroblasts (see Figure 1 b). These were, however, only a small percentage of the total fibroblast population in the dermis. The nuclear morphology of the PCNA-positive fibroblasts appeared highly vesicular and could be easily distinguished from other fibroblast populations in the dermis that were more typical of mature fibroblasts. In the keloid lesion, no proliferating cells could be discerned, the nuclear morphology of the fibroblasts being similar to those in the dermis that were PCNA negative.

In Situ *End Labeling*

In normal skin, positively labeled cells were restricted to the basal layer of the epidermis. In keloid samples, numerous cells showing positive immunoreactivity for DNA strand breaks were observed in the epidermis and dermis (see Figure 2a). In the most acellular, collagenous parts of the keloid, no positive cells were observed (Figure 2b). In most

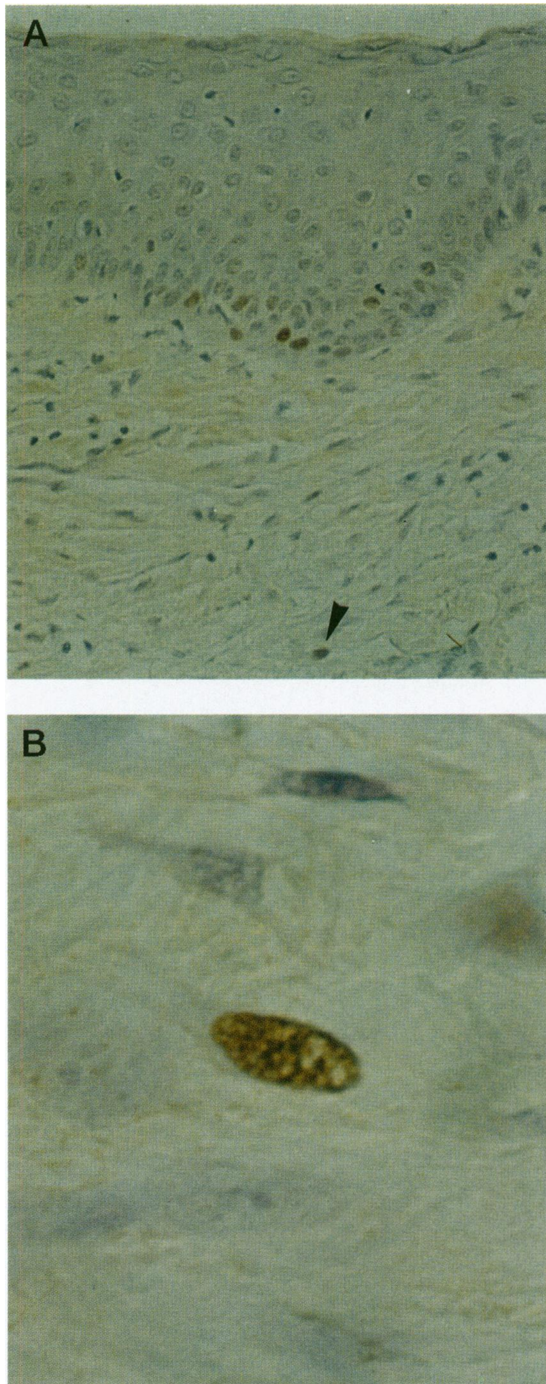

Figure 1. *PCNA-immunopositive epidermal cells in a keloid are shown in* a. *A positive fibroblast in the dermis is also illustrated* (arrowhead). *The specific immunolabeling of a fibroblast is shown at high power in* b. *Magnification,* ×*100* (a) *and* ×*500* (b).

cases, identification of cell type was not possible due to changes in nuclear morphology associated with apoptosis and necrosis. It was, however, possible to distinguish between apoptotic cells and cells undergoing necrosis, both of which were evenly distributed throughout the dermis. Apoptotic endothelial cells of a small proportion of blood vessels in the dermis were observed (see Figure 2c).

Acridine Orange Staining

Few apoptotic keratinocytes were seen in normal human skin (see Figure 3a). In the region of skin juxtaposed to the keloid lesion, numerous apoptotic keratinocytes were seen (Figure 3b), with the number and distribution of positive cells decreasing more distal to the keloid lesion. At the interface between the dermis and keloid, numerous cells with the typical condensed nucleus characteristic of apoptotic cells were seen, although none were observed in the most central part of the keloid (see Figure 3c).

Discussion

The mass of fibrous tissue that constitutes keloids by definition indicates that both pro-mitogenic and prosecretory mechanisms are, or were, involved in their formation. However, although the center of the keloid is highly collagenous, it is avascular with few cells present. In contrast, the interface between the lesion and the dermis contains large populations of lymphocytes and fibroblasts interspersed in a dense capillary network. This inevitably raises the question of what the mechanisms are controlling the cellular evolution and devolution of keloids.

This study has demonstrated that, although numerous lymphocytes and fibroblasts were present in the keloid dermis, only a small proportion of the fibroblast population were PCNA positive. Hence, lymphocyte migration rather than proliferation is mainly responsible for the mass of lymphocytes present in the dermis of a keloid lesion. This suggests that, under the influence of an endogenous antigen, for which keratin and sebum have been postulated as candidates,[5,6] lymphocytes migrate to the area and subsequently drive the fibrotic process.

It has recently been demonstrated *in vitro* that keloid-derived fibroblasts have a greater proliferative capacity than normal dermal fibroblasts.[7] Furthermore, the proliferation profile of fibroblasts in keloids *in vivo* has also been reported.[8] However, differences in the proliferation of cells between the center and the edge of the lesion could not be evaluated. In this study, we have clearly demonstrated that fibroblast proliferation is restricted to the area of dense lymphocyte populations and high blood vessel numbers, with no proliferating cells in the mid-region. In addition, we have recently shown that the area of

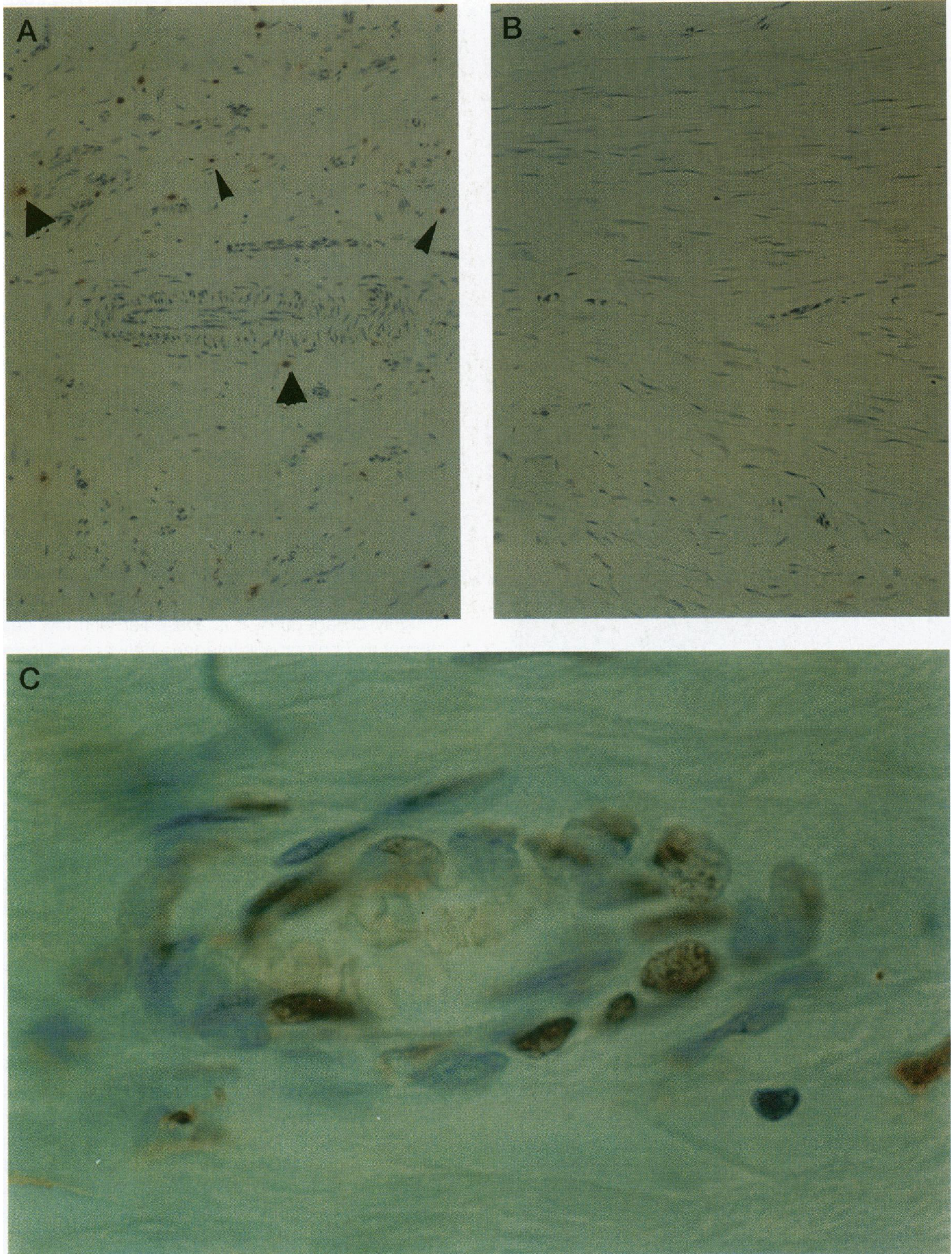

Figure 2. a: *Positive immunoreactive cells labeled for DNA strand breaks in the dermis of a keloid lesion. The* small arrowheads *show the typical condensed morphology of an apoptotic cell. The* large arrowhead *shows the distended appearance of a necrotic cell. In* b: *Mid-region of the keloid. Note the lack of immunoreactivity to DNA strand breaks.* c: *Apoptotic endothelial cells of a blood vessel in the dermis. Magnification,* ×*50* (a *and* b) *and* ×*500* (c).

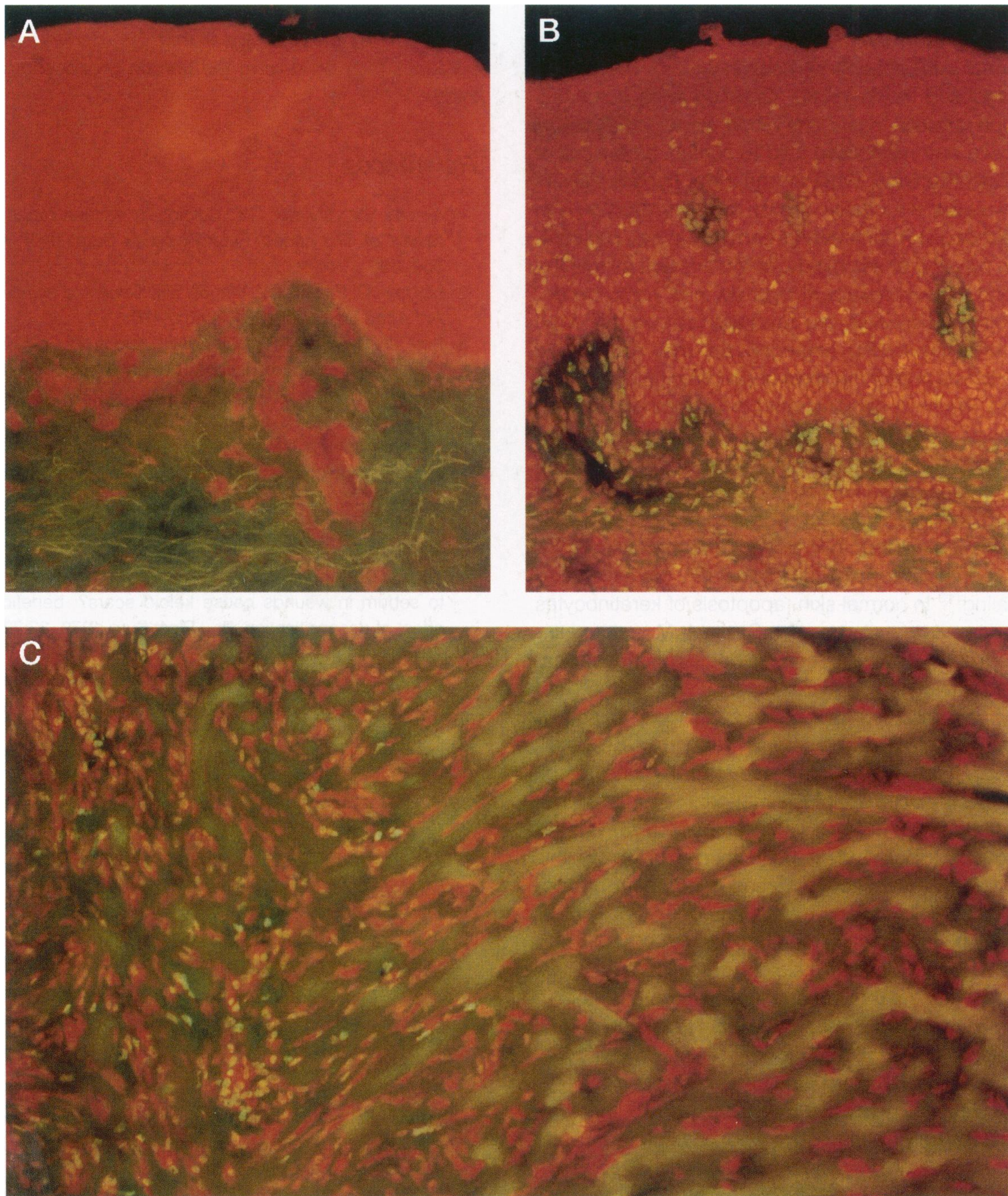

Figure 3. **a:** *Acridine orange staining of normal human dermis.* **b:** *Numerous apoptotic cells in the epidermis and dermis of a keloid.* **c:** *The interface between the dermis and keloid. Note apoptotic cells in the dermis but absence in the keloid. Magnification, ×100.*

dermis juxtaposed to the keloid lesion is rich in numerous cytokines and growth factors whereas the mid-lesion is virtually devoid of any pro-mitogenic factors (N. Brown, personal communication). This clearly indicates that not only is the keloid microenvironment heterogeneous and as such the influence of various factors will be spatially related but also that the subcellular populations are also heterogeneous. This makes interpretation of *in vitro* findings using keloid-derived cell lines difficult.

By light and electron microscopic examination, keloids have long been established to be hypoxic.[9] Hypoxia can stimulate both apoptosis and production of vascular endothelial growth factor,[10] which may be the angiogenic factor in both tumors[11] and inflammation.[12] We have recently demonstrated a

raised incidence of immunoreactive vascular endothelial growth factor in keloids in comparison with normal skin (unpublished observations). The dermis juxtaposed to the keloid lesion contains numerous blood vessels. In fact, the blood vessels are anastomosed and have been said to contain an excess of endothelial cells. Whether the initial hypoxia results in vascular endothelial growth factor production with a subsequent increase in endothelial cell proliferation causing anastomosis, or the anastomosis of the blood vessels results in hypoxia, has yet to be determined. However, the resultant hypoxia, although further potentiating angiogenesis, may also cause apoptosis and/or necrosis of certain cellular populations. We have demonstrated that apoptosis of blood vessels in the keloid dermis occurs whereas no evidence of blood vessel necrosis or apoptosis in the mid-region of the keloid lesion was found. This suggests that blood vessel regression in the keloid lesion is via an apoptotic-dependent process.

Apoptosis is a natural sequela in normal wound healing.[13] In normal skin, apoptosis of keratinocytes may provide a source of IgG and IgM anti-keratin-intermediary-filament autoantibodies.[14] Keloid patients have higher than normal levels of IgG and IgM.[15,16] Whether these are specifically raised to keratin intermediary filaments has yet to be determined. Furthermore, cell-mediated immune attack on cells induces apoptosis, not necrosis.[17,18] This is particularly relevant to keloids, which as stated above are believed to be immune driven. In this study, we have shown increased numbers of apoptotic bodies in the epidermis and dermis of keloids. It may be possible to speculate that the initiating and propagating factor in keloids is that as a result of trauma the normal production of apoptotic keratin bodies may cause a sufficient increase in the normal levels of autoantibodies to keratin intermediary filaments to push toward autoimmune disease.

It was first proposed by Kischer et al[19] that progressive cell degeneration and possibly apoptosis occurred during the transition from the granulation phase to mature scar in keloids and indeed other scars. Here we have confirmed this hypothesis and demonstrated that proliferation, apoptosis, and necrosis occur simultaneously in keloids and that these processes are distinctly compartmentalized. As the keloid matures, apoptosis and necrosis result in selective removal of certain cellular populations resulting in the characteristic avascular fibrotic collagenous lesion, whereas proliferation of fibroblasts in the keloid dermis propagates the fibrosis. Identification of the factors responsible for controlling these processes may lead to novel therapeutic intervention in the treatment of keloids. To this end, we have preliminary findings to suggest that TH_2-derived lymphokines may be driving the fibrotic process in keloids.

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