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Cellular proliferation, differentiation and apoptosis in polyether-polyurethane sponge implant model in mice

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Summary. The integration of implanted material to host organism requires spatial and temporal organization of several cellular processes, such as proliferation, differentiation and apoptosis. Despite the clinical relevance of these processes, there is little information regarding the sequence of such events in synthetic matrices. Here, we present a combination of techniques used to characterize the fibrovascular response in subcutaneous polyether-polyurethane sponge implants in mice at days 4, 7, 10 and 14 postimplantation. The AgNOR technique was modified and used as a surrogate marker for proliferating and activated cells invading the implant. The number of AgNOR-stained cells increased progressively from day 4 (606±76) to day 14 (2146±71) postimplantation. The number of TUNEL-positive (apoptotic index) cells also increased progressively from day 4 (459±40) to day 14 (1157±119) postimplantation. However, the ratio of TUNEL-labeled/proliferating cells had its highest peak in the early phase of the process remaining stable until day 14. Using Picrosirius staining it was shown that thin collagen increased from day 4, peaking at day 10 and falling markedly at day 14, whereas dense collagen increased progressively during the whole period. These experiments hold potential to investigate not only distinct phases of tissue repair induced by synthetic matrices but also to study underlying mechanisms involved.

Key words: Sponge implants, Collagen, AgNOR, TUNEL, Picrosirius

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Introduction

Normal wound healing is a complex biological process governed primarily by the various inflammatory cells that accumulate within the lesion compartment. The sequence of events requires a high degree of coordination and regulation by growth factors and inflammatory mediators. Although the process is continuous, it is commonly referred to as occurring in phases including, coagulation, inflammation, migratory-proliferative processes and remodeling that may overlap to some extent during the whole period (Martin, 1997; Singer and Clark, 1999). During tissue repair all cell types undergo a rapid increase in number, perform their specific activities and later must fall to negligible amount once restoration is completed. The most likely mechanism involved in the resolution of the various phases of tissue repair is through apoptosis (Greenhalgh, 1998; Hall, 1999).

Most of the information regarding the phases and mechanisms of tissue repair has been determined in incisional and open wounds. While these skin wounds offer accurate models of naturally occurring lesions, it is difficult to characterize some features of the newly formed tissue. Very often there are confounding events in adjacent areas of the lesion once the healing process begins. To circumvent this problem and advance the understanding of tissue repair, implantation of synthetic matrices has been used as a framework to induce fibrovascular tissue growth (Viljanto et al., 1981; Davidson, 1998; Andrade, 2001). In addition, at present, there is considerable interest in implantation techniques and scaffolds for tissue engineering for their potential use in controlling tissue regeneration and repair. Therefore assessment of proliferation, differentiation and apoptosis in sponge implants may reveal critical features of the kinetics of the healing process.

The common principle underlying this technique is that injury caused by introduction of the device elicits within the area circumscribed by the implant a response that mimics the stages of wound repair (Andrade et al.,

1987; Ford et al., 1989; Walsh et al., 1996; Kyriakides et al., 2001; Opalenik and Davidson 2005). This model provides most times, a chronically inflamed environment in which several components of the fibrovascular tissue induced by the implants have been characterized. Sponge model has been used to study angiogenesis, inflammation, cytokine and chemokine production, as well as the development of vasoactive regulatory systems and pharmacological reactivity of the neovasculature in a variety of normal and pathological conditions (Ford et al., 1989; Andrade et al., 1992; Walsh et al., 1996; Barcelos et al., 2004; Belo et al., 2004).

In this study, cellular proliferation, differentiation and apoptosis were assessed in a sponge model of inflammatory angiogenesis in mice. We have established a range of techniques (AgNOR as marker for proliferation and cellular activity; Picrosirius staining for collagen determination as marker for cellular differentiation and TUNEL for apoptosis). These techniques have been extensively used in many different tissues to characterize cellular proliferation and regression in several healing processes (Aubele, 1994; Kerr et al., 1994; Tuccari et al., 1999; Kesler et al., 2000). Now they have been adapted to characterize the kinetics of sponge-induced wound healing, providing a new approach to investigate underlying mechanisms involved in processes of implant-host interface.

Material and methods

Male Balb/c mice 7-8 weeks were used in these experiments.

Preparation of sponge discs and implantation

Polyether-polyurethane sponge discs, 5 mm thick x 8 mm diameter (Vitafoam Ltd., Manchester, UK) were used as the matrix for fibrovascular tissue growth. A 12 mm length polyvinyl tubing (PE 20, Biovida, Brazil) was attached with silk sutures (Ethicon Ltd, UK) to the center of each disc in such a way that the tube was perpendicular to the disc face. Its open end was sealed with a removable plug. The cannulated sponge discs were soaked overnight in 70% v/v ethanol and sterilized by boiling in distilled water for 15 minutes before the implantation surgery. For that, the animals were anesthetized with 2,2,2-tribromoethanol (1 mg kg^{-1}; i.p. Aldrich/USA), the dorsal hair shaved and the skin wiped with 70% ethanol. The cannulated sponge discs were aseptically implanted into a subcutaneous pouch, which had been made with curved artery forceps through a 1 cm long dorsal mid-line incision. The cannula, used to keep the implants in place, was exteriorized through a small incision in a subcutaneous neck pouch (Andrade, 2001). The animals were housed individually and provided with chow pellets and water *ad libitum*. The light/dark cycle was 12:12 h with lights on at 7:00 am and lights off at 7:00 pm. Efforts were made to avoid any unnecessary distress to the animals. Housing and anesthesia concurred with the guidelines established by our local Institutional Animal Welfare Committee.

Histological analysis and staining

The implant-bearing mice were killed by cervical dislocation at 4, 7, 10 and 14 days post-implantation and the sponge discs carefully removed, dissected free from adherent tissue, weighed and fixed in formalin (10% w/v in isotonic saline). For each time point 7 implants from 7 different animals were examined. Sections (5µm) were stained and processed for light microscopic studies and morphometric analysis. Hematoxylin and eosin (HE) and Shorr staining were used for determining the implant fibrovascular area. AgNOR, a silver staining technique, commonly used to detect changes in nucleolar organizer regions (Ploton et al., 1986; Aubele et al., 1994), was modified and adapted to the sponge implant material as a marker for cellular activity/cellular proliferation. The AgNOR positive cells were counted to estimate the proliferation/activation rate. Picrosirius staining followed by polarized-light microscopy was used to visualize and determine collagen fibers (Junqueira et al., 1979).

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescence nick end labeling (TUNEL) method (Gavrieli et al., 1992) in paraffin-embedded sponge implant sections mounted on glass slides. The TUNEL positive cells were counted to estimate the apoptotic event. An apoptosis/proliferation ratio was achieved by dividing the total number of TUNEL positive cells to the total number of AgNOR positive cells. To perform morphometric analysis, images of cross-sections obtained from 20 fields per slide (8,533 μm^2/field) were obtained with a planapochromatic objective x40 in light microscopy (final magnification =1000x). The images were digitized through a JVC TK-1270/JGB microcamera and transferred to an analyzer (Kontron Eletronics, Carl Zeiss – KS300 version 2).

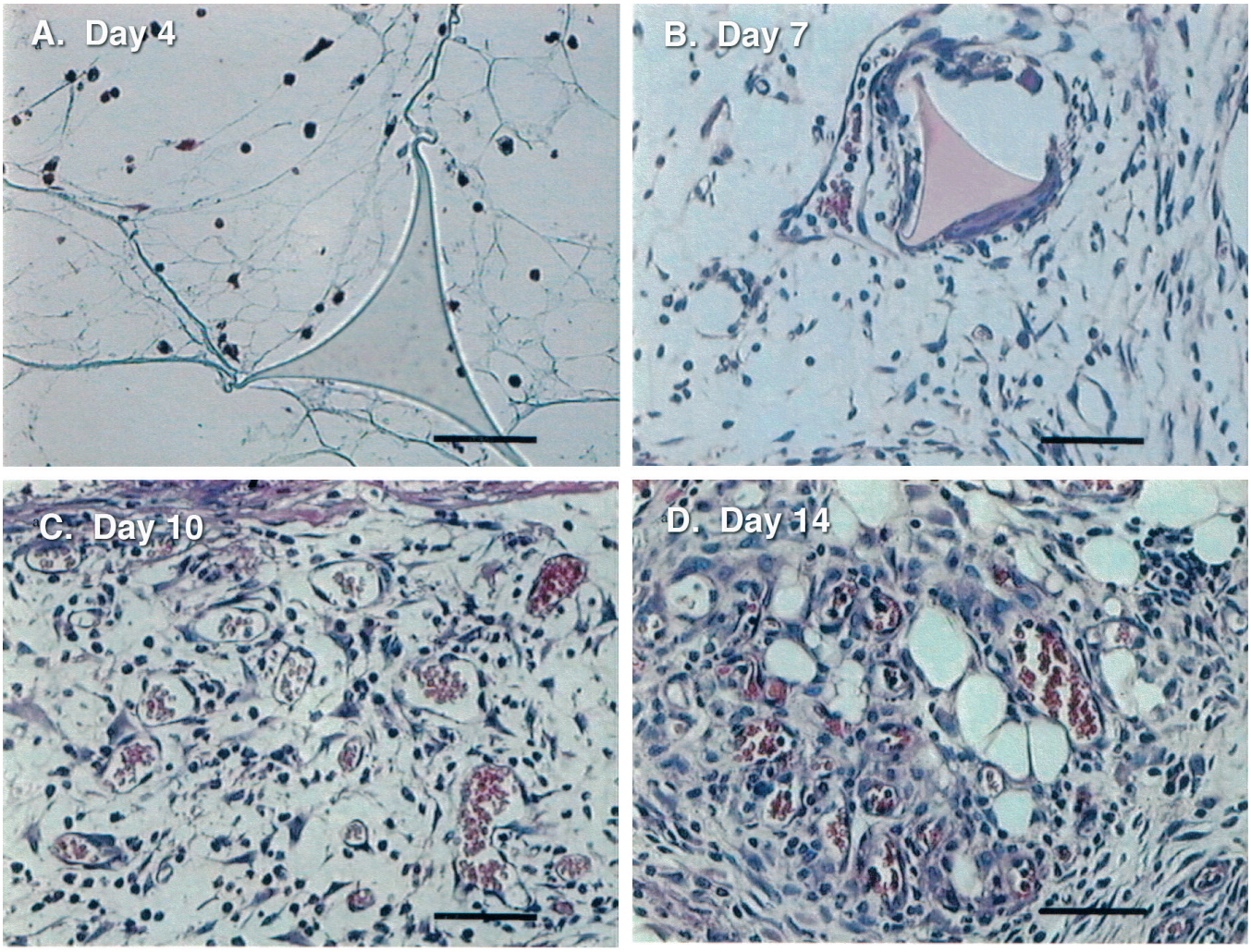
Statistical analysis

Results are expressed as means±SEM. Comparisons between groups (N=7 mice per group) were made using one-way analysis of variance (ANOVA) followed by Newman-Keuls correction factor for multiple comparisons as a post –hoc test. Statistical analysis was performed using Graph-Pad Prism 2.01. Differences between means were considered significant when p values were <0.05.

Results

Histological assessments

Subcutaneous implantation of cannulated sponge discs in mice induced a wound repair response causing



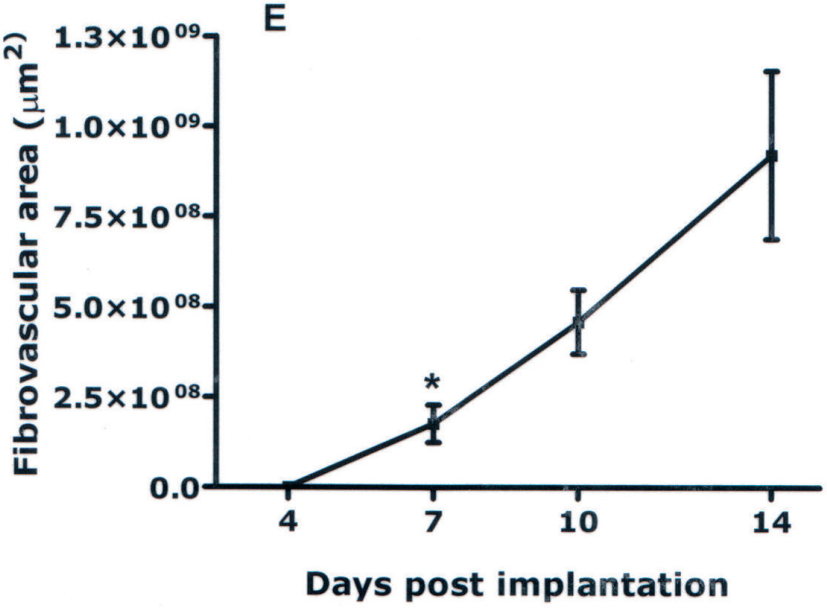


Fig. 1. Kinetics of representative histological sections (HE, 5 µm) of fibrovascular granulation tissue induced by sponge implants at days 4 **(A)**, 7 **(B)**, 10 **(C)** and 14 **(D)**. The pores of the sponge matrix, seen as triangular shapes, are initially filled with a fibrinous network and numerous polymorphonuclear leukocytes. The matrix is progressively infiltrated by inflammatory cells, spindle-shaped like fibroblasts, endothelial cells and blood vessels embedded in a dense organized matrix. Kinetics of the morphometric analysis of the fibrovascular area (μm^2) corroborated the histological findings **(E)**. Arrows, blood vessels. Bar: 100 µm.

the synthetic matrix to be filled with fibrovascular tissue as shown by HE staining. The sponge implants were progressively infiltrated by fibrovascular stroma localized near the implant surface at day 4, and occupying the entire sponge by day 14. This granulation tissue was composed of a dense inflammatory infiltrate, with macrophages, spindle-shaped like fibroblasts and proliferating endothelial cells forming microvessels (Fig. 1A-D). The morphometric area (μm^2) occupied by the fibrovascular tissue grew throughout the experiment and corroborated the histological assessment (Fig. 1E).

After determining the kinetics of the fibrovascular tissue infiltration over a 14-day period post implantation, we have modified and standardized the AgNOR technique as an index of cell proliferation and activation in the sponge implants. All silver-stained sections showed an adequate staining intensity. The AgNOR staining pattern in the cells was characterized by the presence of aggregates, clustered in irregularly shaped collections. The silver dots appeared as dark structures on a grey nuclear background. Representative microphotographs of stained cells are shown in Fig. 2. The number of AgNOR-stained cells, as determined by morphometric analysis, showed an increase from 606±76 cells at day 4 to 2146±270 cells by day 14 after implantation. To further analyze the turn over pattern of the cell population within the fibrovascular tissue induced by the sponge implants, the total number of TUNEL-positive cells was determined in the four intervals after implantation. Representative microphotographs (Fig. 3) of apoptotic cells show dark-brown TUNEL positive nuclei with other morphological features of cellular death (anoikia, cellular shrinkage, condensed chromatin). Similar to proliferation data,

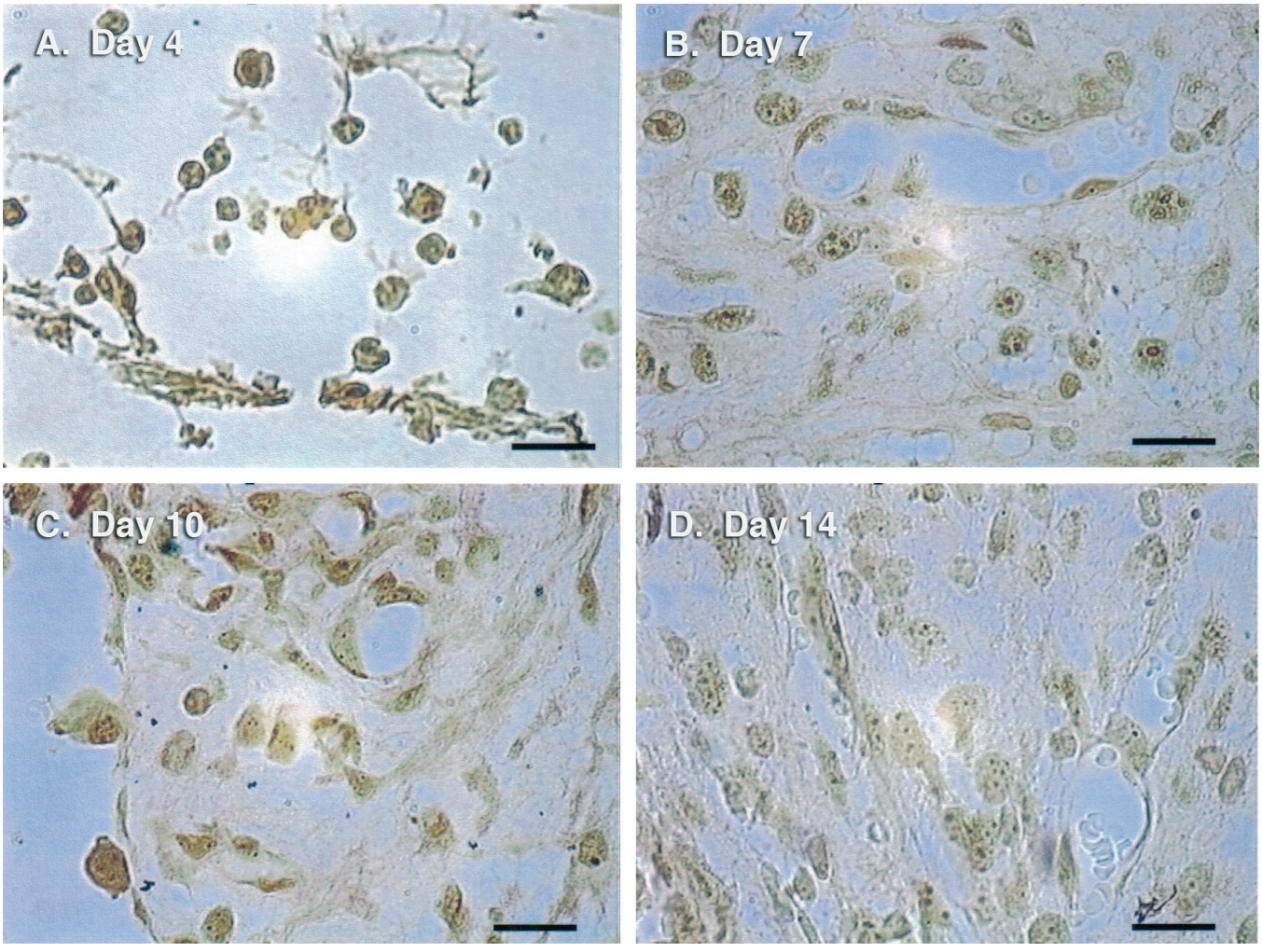


Fig. 2. Silver stained histological sections of sponge implants. Images are representative of seven mice from each time point. The AgNORs in the implant sections are seen as black dot aggregates in the nucleoli of the inflammatory, endothelial and spindle-shaped cells throughout the whole process. Bar: 10 μm.

apoptosis increased progressively from day 4 until day 14 (Fig. 4A,B). It is interesting to note that although both cytological parameters showed progressive increase, the apoptosis/proliferation ratio was much higher in the early phase of the process (day 4), decreased at day 7 and remained stable afterwards (Fig. 4B). To assess cellular differentiation, densitometric analysis of collagen content was determined in the implants using Picrosirius staining and polarized light microscopy. Dense collagen increased progressively with time, whereas thin collagen increased until day 10 and fell markedly at day 14 (Figs. 5, 6).

Discussion

In response to tissue injury, inflammatory cells and their products initiate and maintain a host response designed to repair the damaged tissue. In our study, the sponge implant was the stimulus to trigger the cascade of inflammatory events that culminated in the formation of a fibrovascular tissue. The sequence of observed events - inflammation, angiogenesis, synthesis of a provisional extracellular matrix, collagen deposition and apoptosis - was similar to that observed in natural cutaneous wound healing reported in earlier studies (Greenhalgh, 1998; Singer and Clark, 1999). Previous work in our laboratory has established the kinetics of angiogenesis, inflammation, cyto/chemokine production in sponge implants (Machado et al., 2000; Barcelos et al., 2004; Belo et al., 2004; Ferreira et al., 2004). Here, by using a range of techniques to evaluate cellular proliferation, differentiation and apoptosis, it was possible to demonstrate that in the sponge implants these events are distinct, although overlapping to some extent.

The silver-staining technique has been used to detect argyrophilic proteins at the nucleolar organizer regions

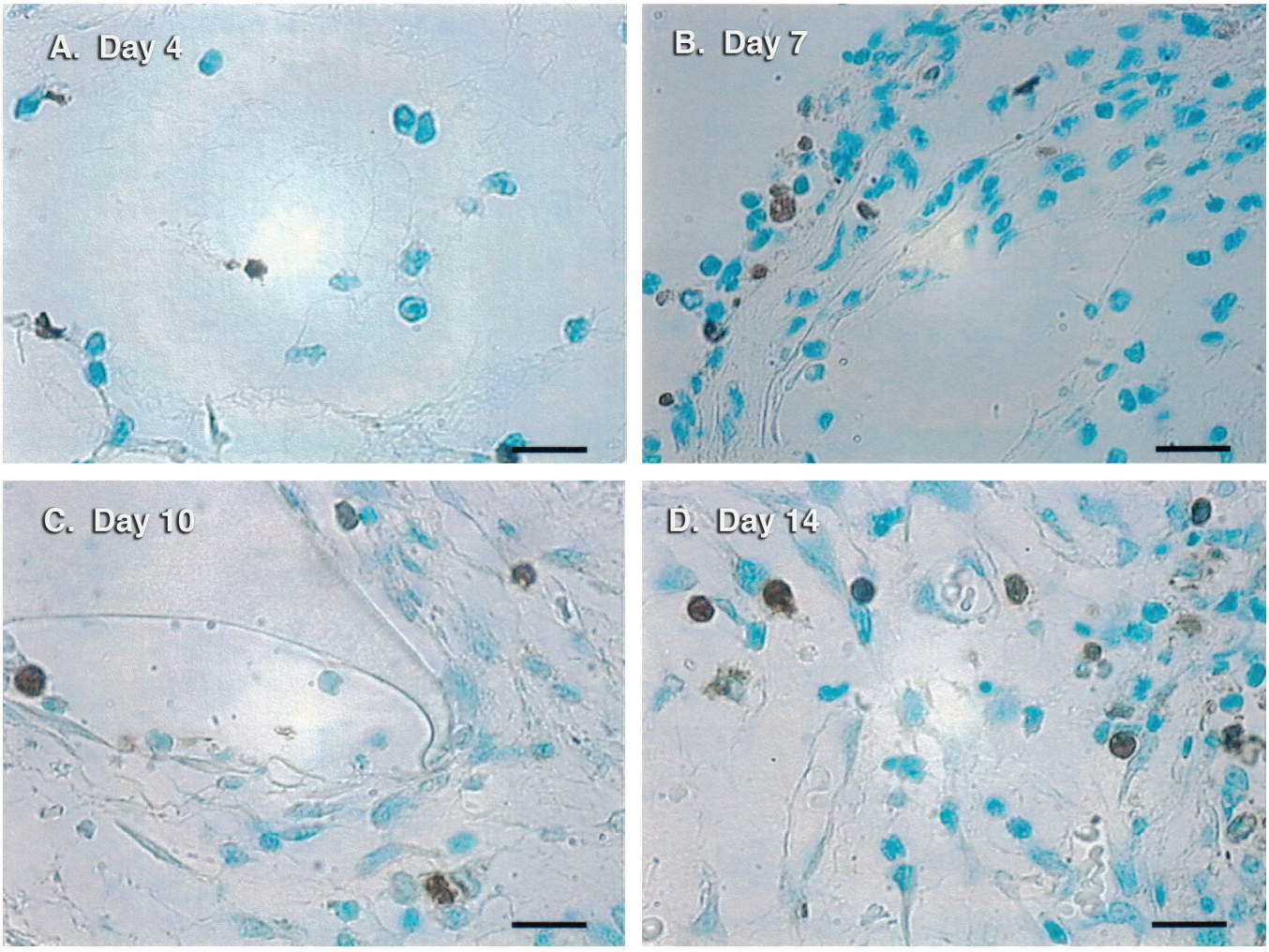


Fig. 3. Representative histological sections (TUNEL staining) of the fibrovascular tissue induced by sponge implants at days 4, 7, 10 and 14 post-implantation showing apoptotic cells (**A, B, C and D**). Images are representative from seven mice for each time point. Bar: 10 μm.

(NORs), allowing the determination of proliferative rate in several pathological conditions (Derenzini et al., 1990; Muscarà et al., 1997; Tuccari et al., 1999). In wound healing, the morphometric analysis of AgNORs has been used to characterize the evolutive phase of lesions (Godoy et al., 2000). Furthermore, evidence exists that in nonproliferating cells the number of NORs may be considered a marker of protein synthesis activity (Jozsa et al., 1993). The AgNORs in the implant sections were seen as black dot aggregates in the nucleoli of the inflammatory, endothelial and spindle-shaped like cells throughout the whole process. To date, no studies have been performed using AgNOR technique to determine cellular activation and proliferation rate in wound healing models induced by synthetic implants. Because the silver-staining procedure is a simple, reliable and inexpensive technique and has been described to correlate well other proliferation markers, Ki67, PCNA (Löhr et al., 1997; Slowinska-Klencka et al., 2004), we consider that valuable information may be obtained in other implant material using this technique.

Many defense mechanisms employed by inflammatory cells are potentially deleterious to host tissues. Thus, it is important that granulocytes are effectively and rapidly destroyed concomitant with the removal of an inflammatory stimulus to avoid excessive tissue damage or chronic inflammation. Apoptosis, a form of programmed cell death, is considered the main mechanism by which inflammatory cells are removed

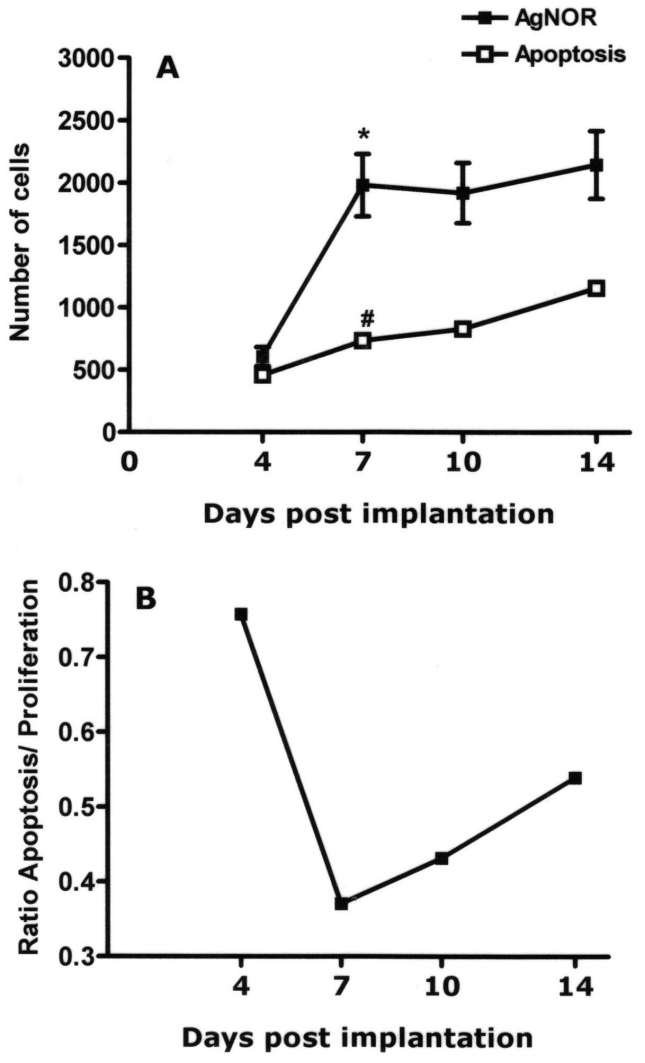


Fig. 4. Patterns of cell proliferation and apoptosis **(A)** and the ratio apoptosis/proliferation **(B)**. Both activities increased progressively during the whole period. The apoptotic peak occurred at day 4, the AgNOR expression was highest after 7 days. Data represent mean± SEM from 7 animals for each interval. * p<0.05 compared with day 4 (AgNOR); # p<0.05 compared with day 4 (apoptosis).

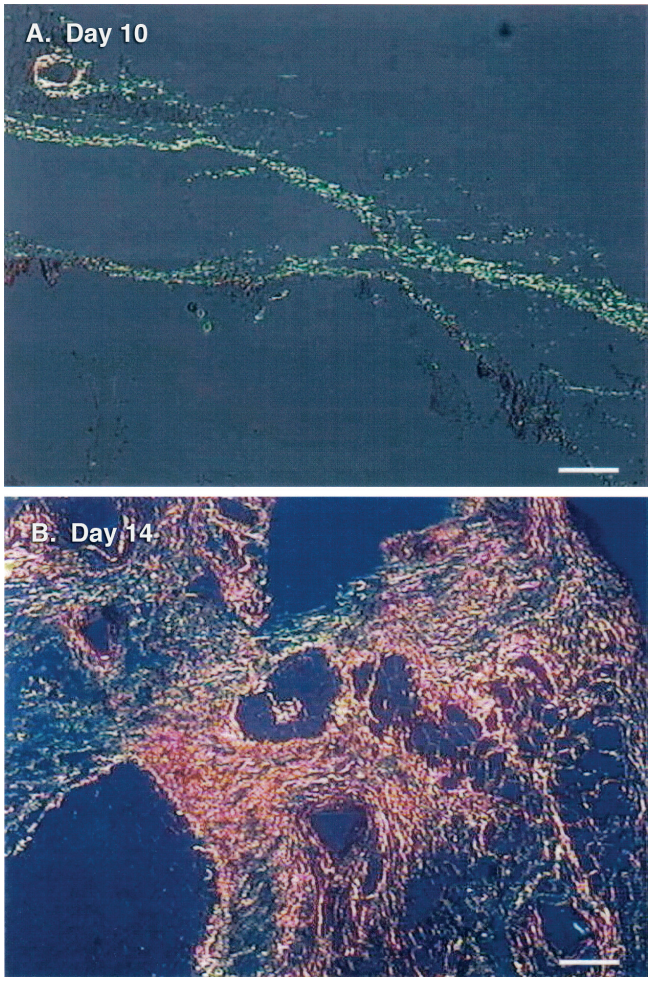


Fig. 5. Representative histological sections (Picrosirius staining) of the fibrovascular tissue induced by sponge implants day 10 **(A)** and 14 **(B)** showing the pattern of type III (green, **A**) and type I collagen (red, **B**). Images are representative from seven mice for each time point. Bar: 10μm

from injured sites (Brown et al., 1997; Greenhalgh 1998). As detected by TUNEL staining, apoptosis was present at all time points, indicating an active cellular turnover within the pores of the sponge. However, considering the apoptosis/proliferation ratio, this cell death was proportionally more pronounced at day 4. In the nonsuppurative acute inflammation, apoptosis plays an important role in removing leukocytes from the inflammatory site and also in controlling inflammatory processes in open and incisional wounds (Greenhalgh 1998). This may be associated with change in the inflammatory profile when other cell types invade the wound compartment (Brown et al., 1997). Indeed, in the sponge implant, other cells and newly formed vascular structures were present from day 7 onwards (spindle-shaped like fibroblasts, endothelial cells, macrophages, blood vessels). In our studies we observed that after the peak at day 4, the apoptosis/proliferation ratio decreased by day 7 and remained stable until day 14 post implantation. Interestingly, in rat skin incisional wound the TUNEL index increased rapidly from day 1, peaking on day 7 after injury (Akasaka et al., 2004). It is possible that the occurrence of apoptosis depends on timing and on different intensities of injuries. It is not surprising that in synthetic implants, apoptosis remains for as long as the stimulus persists, probably as a turnover mechanism and also as a control against excessive amplification of inflammatory processes.

Collagen deposition in wound compartment is a hallmark of tissue repair, giving to incisional and open wounds much of the strength lost after injury. In our experiments, Picrosirius red staining showed two distinct patterns of collagen within the sponge matrix. Collagen deposition increased progressively from day 4 until day 14 and fibers became thicker, changing from green to yellow/red. It has been presented that thick fibrils consistis of type I collagen and thin type III collagen (Kolodgie et al., 2002).This indicates fibroblast differentiation, an important event in wound healing (Grinnell, 1994; Demouliere et al., 1997). This profile of collagen deposition in the sponge implants is consistent with previous work in other implanted matrix and in open and incisional wounds (Kurkinen et al., 1980; Viljanto et al., 1981; Gabbiani, 2003; Opalenik and Davidson, 2005).

In conclusion, the sponge implant model elicited a wound repair response in which a number of overlapping phases could be identified and quantified using a range of histomorphometric techniques. It is central for implant materials or scaffolds for tissue engineering to be able to host cells to rebuild functional tissue substitutes at the same time as modulating the natural healing process. The experimental approach described here holds potential to investigate important features of new materials such as implants or scaffolds for tissue engineering and engraftment.

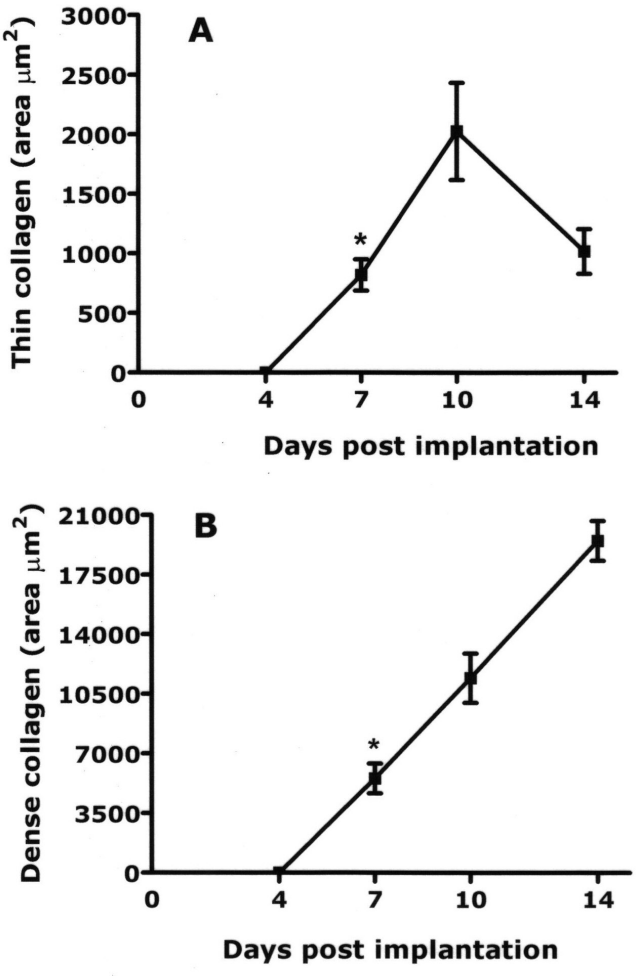


Fig. 6. Morphometric analysis of the collagen deposition showing the peak of type III collagen (**A**, green area) at day 10 and progressive increase of type I collagen (**B**, red area) during the 14-day-period post implantation. Data represent the mean±SEM from 7 animals for each time interval. * p<0.05 compared with day 10 **(A)**, * p<0.05 compared with day 4 **(B)**.

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