Inflammatory response to a porcine membrane composed of fibrous collagen and elastin as dermal substitute

B. KLEIN[1], R. SCHIFFER[1], B. HAFEMANN[2], B. KLOSTERHALFEN[3], G. ZWADLO-KLARWASSER[4]
[1]Interdisciplinary Center of Clinical Research BIOMAT (IZKF BIOMAT), [2]Clinic of Plastic Surgery, Hand and Reconstructive Surgery, [3]Institute of Pathology, [4]Department of Dermatology, Medical Faculty, RWTH Aachen, Germany

The inflammatory response to a collagen/elastin membrane was studied by measuring the expression of cytokines and function associated antigens in human macrophages. Additionally the angiogenic and inflammatory activity in the chorioallantoic membrane of the chick embryo (CAM-assay) was investigated. Macrophages cultured on the membrane expressed IL-1β mRNA as early as after 4 hours. During prolonged culturing IL-1β mRNA levels decreased. Messenger RNA for IL-8 was detectable over the whole culture period. The anti-inflammatory cytokine IL-10 was expressed up to one day only. Phenotypic analysis revealed a decrease in the number of chronic inflammatory 25F9 positive macrophages not migrating into the membrane but a presence of these cells together with the acute inflammatory 27E10 macrophages within the membrane whereas the anti-inflammatory subtype RM3/1 was absent. In the CAM-assay the membrane stimulated angiogenesis and induced the formation of granulation tissue. Histological analysis showed that the membrane was infiltrated with macrophages, fibroblasts and endothelial cells and locally with granulocytes. These data show that the collagen/elastin membrane causes activation of macrophages, angiogenesis and the formation of inflammatory tissue. Although these processes are essential for wound healing the type of inflammation points to a chronic process which might counteract an efficient scar formation.

1. Introduction

Until now the reconstruction of severe skin defects by a fully functional artificial skin is still an unsolved problem because both epidermal as well as dermal equivalents are required. While a neoepidermis can be acchieved by transplantation of *in vitro* expanded keratinocytes [1, 2], at the present no artificial dermal replacement exists which is qualitatively comparable with native autogenic dermis. The use of collagenous matrices was introduced as early as around 1980 as an alternative method of dermal skin replacement, e.g. in cases of severe and extensive burns [3]. These scaffolds are based on the assumption to simulate a three-dimensional molecular structure serving as template to initiate cellular colonization and normal wound healing processes [4, 5].

Wound healing is defined as the restoration of the continuity of living tissue and is an integrated response of several cells to injury. Important steps in wound healing include an inflammatory response and neovascularization [6]. It is well established that macrophages are pivotal cells in the initiation and regulation of inflammation and of healing processes by releasing numerous mediators regulating cellular growth and activities [7–9]. Moreover, macrophages have been recognized to be a central cell type in mediating angiogenesis [6, 7, 10]. However, suitable models for evaluation of the ability of artifical dermal substitutes to stimulate inflammation or to promote angiogenesis are still missing. In this study we applied an *in vitro* culture system to measure the inflammatory response to a novel porcine collagen/elastin membrane, meant to be used as dermal substitute, at the level of macrophages [2]. Moreover, the chorioallantoic membrane assay (CAM) of the chick embryo was employed as alternative *in vivo* model widely used for examination of angiogenesis but also as model for inflammation [11–14].

2. Materials and methods

A membrane of porcine origin was used, which had been subjected to very gentle cleansing procedures in order to retain an acellular, structurally native matrix (Bioplex Medical B.V., Vaals, The Netherlands). The matrix is mainly composed of collagen (70%) and elastin [15]. Teflon (Biofoil, Heraeus) was employed as a cell culture control.

2.1. Cells

Monocytes were obtained from buffy coats of healthy donors by two-step density gradient centrifugation using Ficoll-Paque and Percoll [16] resulting in a purity of at least 80% as estimated by staining with CD14 antibody. For investigation of phenotype modulation they were cultured at a density of 2×10^6/ml in RPMI 1640 (Gibco BRL, Germany) containing 10% heat-inactivated human serum (pooled from three donors) in direct contact with the membrane. Monocytes precultivated for two days on hydrophobic Teflon foil were used for analysis of cytokine mRNA expression in order to reduce the unspecific activation due to the isolation procedure [17] and are therefore referred to as macrophages. Lipopolysaccharide (1 μg/ml Lps, Sigma, Deisenhofen, Germany) was used as an inducer of cytokines in cells cultured on Teflon. After 4 h, 1, 2, 4, 6 or 7 days the cells were harvested and processed for functional analysis as described below.

2.2. RT-PCR analysis for detection of mRNA

Messenger RNA was extracted from 3×10^6 cells with the Oligotex Direct mRNA-purification kit (Qiagen, Hilden, Germany) using the mRNA-enrichment protocol as described in Kuribayashi *et al*. [18]. Reverse transcription (RT) and PCR were performed with the Gene Amp RNA PCR-kit (Perkin Elmer, Weiterstadt, Germany) according to the manufacturer's instructions. Competitive PCR was carried out using primers for each cytokine together with primers for β-actin in one sample. The sequences for the primer pairs for IL-8 were designed according to the protocol from Clontech (Heidelberg, Germany), for IL-1β and IL-10 as well as for the internal standard β-actin according to published sequences [19–21]. Amplification was performed with 35 cycles of 1 min denaturation at 93 °C, 1 min annealing at 62 °C for IL-1β and IL-8 and 66 °C for IL-10 and 1 min extension at 72 °C. Amplification was terminated with an extension step of 5 min duration after the last cycle. PCR products were separated on 1.8% agarose gels and stained with ethidiumbromide.

2.3. Flow cytometric and histochemical analysis of the macrophage antigens 27E10, RM3/1 and 25F9

Samples of cells (0.5×10^6) obtained from culture supernatants covering the membrane were stained with the monoclonal antibodies 25F9, 27E10 and RM3/1 [16, 22, 23] using an indirect immunofluorescence technique and FACS can flow cytometer as reported earlier [16]. For determination of the macrophage subtypes immigrated into the membrane cryostate sections were made and stained with the monoclonal antibodies mentioned above and the CD68 antibody KI-M7 (BMA, Augst, CH) using an indirect immunoperoxidase method with gamma-aminoethylcarbazole as substrate [16].

2.4. CAM-assay

Fertilized hen eggs from the Bückerhof (Aachen, Germany) were incubated at 37 °C for 5 days (angiogenesis) or 6 days (inflammatory tissue). After these periods a triangular window was made in the shell and a disk (∅ 12 mm) of the sterile membrane was placed on the chorioallantoic membrane. The opening in the shell was sealed to prevent dehydration and the eggs were incubated at 37 °C for another 7 or 9 days (angiogenesis) or 8 days (inflammation). At the end of these periods the membrane was either fixed *in situ* using buffered 2% formalin, excized and mounted on a slide, or the matrix together with the underlying granulation tissue was dissected and processed for routine histology. Quantitation of blood vessels was carried out by counting blood vessels at 25 fold magnification in three non-overlapping areas at 25 fold magnification. The average of the numbers of small vessels (20–40 μm) of these areas was taken as vacularization index. In negative controls no material was implanted.

2.5. Data presentation

Data obtained from RT-PCR analysis were expressed semiquantitatively as given in Tables I–III. All other data were means ± SD. Statistical analysis was carried out using the Student's *t*-test for unpaired samples. Values were considered to be statistically significant if $p < 0.05$.

3. Results

3.1. Cytokine mRNA expression

RT-PCR analysis revealed that IL-1β mRNA was detectable as early as 4 h after culturing macrophages on the collagen/elastin membrane (Fig. 1a). Teflon used as non-activating control material [17] did not lead to a similar IL-1β mRNA expression particularly after 4 h. The amount of mRNA elicited by the membrane was comparable to the effect of Lps. During prolonged culturing the majority of IL-1β mRNA levels decreased, but were still present after 7 days and in two of six experiments even increased. IL-8 mRNA was present over the whole culture period and again similarly expressed as in cells treated with Lps (Fig. 1b). IL-10 mRNA was found to be strongly expressed up to one day in culture but only slightly if at all at later stages (Fig. 1c). In contrast, macrophages cultured on the control material Teflon revealed increasing expression of IL-10 mRNA. The semiquantitative summary of results using cells from different blood donors are given in Table IV.

3.2. Expression of macrophage antigens

Analysis of phenotypic changes in macrophages which did not migrate into the membrane revealed that compared to Teflon the membrane caused a small increase in the number of inflammatory 27E10- and a weak decrease in RM3/1 anti-inflammatory macrophages but a statistically significant reduction of the portion of 25F9 macrophages (Table I). These effects were, however, only seen after 7 days.

On the other hand macrophages within the membrane expressed the acute inflammatory 27E10 and the chronic 25F9 antigens but not the anti-inflammtory phenotype RM3/1 (Table II). Regarding the time course of the migration into the membrane, macrophages were found

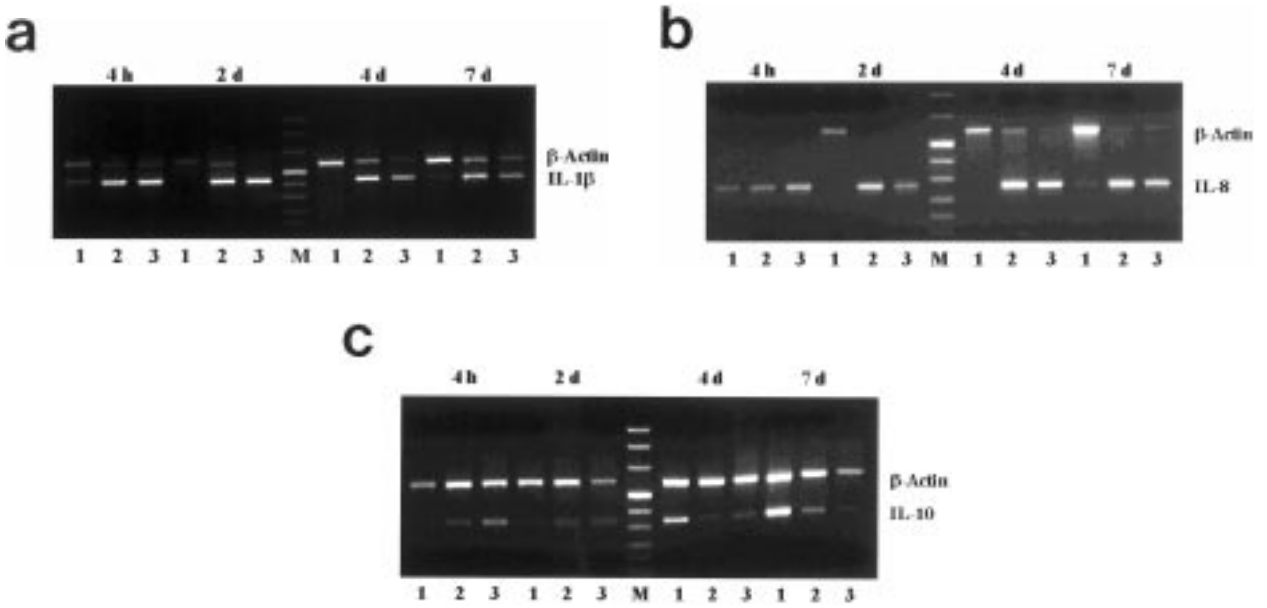

Figure 1 Detection of IL-1β (a), IL-8 (b) and IL-10 (c) mRNAs by competitive RT-PCR in monocytes precultured for 2 days on Teflon foil and than cultured for 4 hours, 2, 4 and 2 days on the collagen/elastin membrane or Teflon foil. Ethidium bromide staining of 1.8% agarose gels. For competitive RT-PCR primers for both, the respective cytokine and β-actin were added in each sample. M, DNA molecular weight marker; lane 1, Teflon foil; lane 2, collagen/elastin memebrane; lane 3, Teflon foil + Lps (1 μg/ml).

to be present in the upper third after 3 days of culture and in about half of the membranes after 5 days. After 7 days the cells were found throughout the membrane.

3.3. Angiogenesis and inflammatory tissue (CAM-assay)

Macroscopical evaluation clearly showed a directed growth of vessels towards the collagen/elastin membrane (Fig. 2a). In about half of the experiments the membrane was completely enclosed by the chorioallantoic membrane (Fig. 2b). Moreover, the membrane caused capillary growth as measured by an increase in the number of small ($> 20\,\mu m$) blood vessels compared to the controls (Table III).

The collagen/elastin membrane also caused the formation of inflammatory tissue in the CAM-assay. Histological examination revealed that the membrane was infiltrated with cells consisting mainly of macrophages, fibroblasts and endothelial cells (Fig. 2c). Locally also granulocytes were found as described earlier [24].

4. Discussion

The collagen/elastin membrane investigated in the present study was designed to retain an acellular, structurally native matrix in order to transmit an adequate message for the formation of a neodermis [15]. A key problem in using such a scaffold is to achieve a quality which can be controled by *in vitro* or *in vivo* assays. Since wound healing is necessary to promote cellular colonization and to remodel the matrix, models stimulating key events in healing processes, e.g. inflammation and neovascularization are required. Therefore we used an *in vitro* culture system to evaluate the inflammatory response of macrophages, cells which are known to be essential in the regulation of inflammatory and healing processes. Moreover, the chorioallantoic membrane assay of the chick embryo was employed as a simple *in vivo* model to study the angiogenic response as well as induction of inflammatory tissue.

Our data indicate the collagen/elastin membrane induces IL-1β- and IL-8 mRNA expression and down-regulates IL-10 mRNA in macrophages. These cytokines

TABLE I FACS analysis of antigen expression of monocytes cultured for 7 days on the collagen/elastin membrane or Teflon foil

Antigen	% positive macrophages Teflon	Collagen	Fluorescence Intensity Teflon	Collagen
27E10	10 ± 4.0	17 ± 10	52 ± 40	124 ± 40
RM3/1	56 ± 10	36 ± 9	201 ± 84	187 ± 85
25F9	49 ± 13	31 ± 10*	85 ± 25	10 ± 6*

TABLE II Immunohistological analysis of antigen expression of macrophages migrated into the collagen/elastin membrane (% of CD68 positive cells, means of four experiments)

Day of culture	27E10	RM3/1	25F9
3	13	0	0
5	32	0	4
7	23	0	25

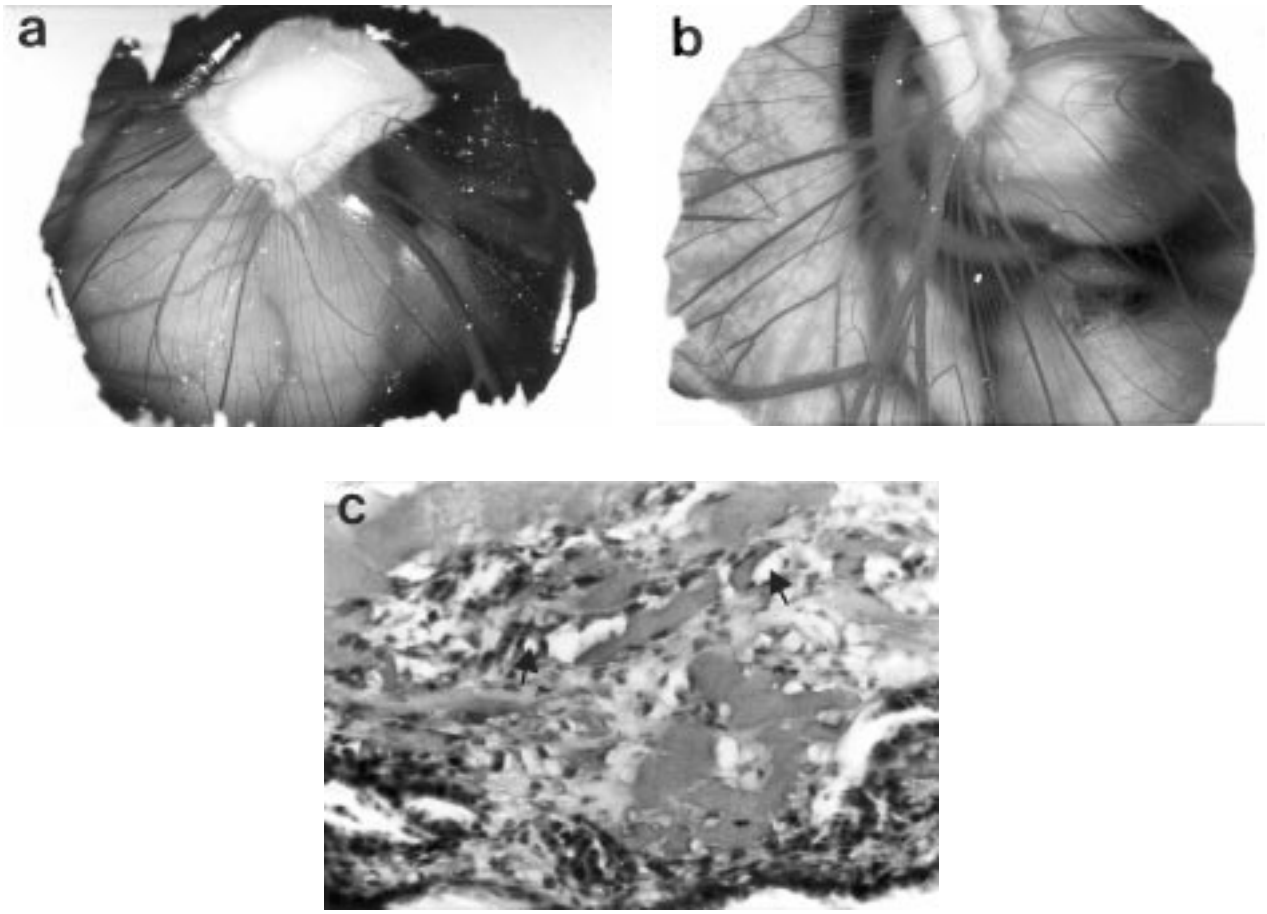

Figure 2 Photographs of the chorioallantoic membrane (CAM) of the chick implanted with collagen/elastin membrane. (a) Directed growth of blood vessels to the collagen/elastin membrane 7 days after implantation. (b) Integration of the collagen/elastin membrane into the CAM after 9 days. (c) Paraffin section of the inflammatory tissue induced by the membrane (H&E staining, 250 ×. Contact area of the collagen/elastin membrane (lower part) with many infiltrating cells. Within the membrane small capillaries (arrows) and scattered inflammatory cells are seen.

belong to the network of mediators participating in the induction and effector phases of all immune and inflammatory responses. IL-1 is the prototype of the proinflammatory cytokine which affects nearly every cell type [25]. The major functions of IL-1 include the induction of the specific lymphocyte reaction, of the acute phase response as well as the activation of granulocytes and endothelial cells. In contrast to the pleiotropic effects of IL-1, the chemokine IL-8 mainly exerts a rather narrow action profile e.g. attraction and activating of granuloctes thereby promoting the unspecific immune response [26]. In contrast to these proinflammatory activities, IL-10 is associated with anti-inflammatory and immunosuppresive properties mainly due to its blockade of the proinflammatory cytokines [27].

The cytokine pattern expressed upon contact with the collagen/elastin membrane strongly suggests an activation of the macrophages leading to an inflammtory response. This assumption is further supported by the finding that the membrane induced cytokine expression is qualitatively similar to the effect of Lps which simulates the macrophages reaction to an infectious agent.

Our data also show that the membrane affects macrophage phenotype by modulating the expression of the function associated macrophage antigens 27E10, RM3/1 and 25F9. These antigens have been found to be present at different stages of macrophage differention [16, 22, 23]. Moreover, the antigens define macrophage subsets which appear in distinct phases during inflammation. For example, cells bearing the 27E10 antigen – a heterodimer of the calcium-binding proteins MRP8 and MRP14 – were only found in acute inflammation and produce large amounts of proinflammatory cytokines such as IL-1β [22, 28]. In contrast, macrophages

TABLE III Effect of the collagen/elastin membrane on angiogenesis measured by the numbers* of small (20–40 μm) vessels (capillaries) compared to untreated controls

Material	Day 7	Day 9
Controls	$34 \pm 5.5(24)$	$36 \pm 7.2(19)$
Collagen	$40 \pm 8.7(16)^{\dagger}$	$44 \pm 7.0(12)^{\dagger}$

*The values represent mean ± SD and the number of eggs (living embryos) indicated in brackets.
†$p < 0.05$.

TABLE IV Summary of the results of cytokine mRNA expression in macrophages from different donors ($N = 4$-6) cultured for the indicated periods on the collagen/elastin membrane or Teflon foil

	Culture period				
	4 h	1 days	2 days	4 days	7 days
IL-1β					
Collagen	++	++	++	+	+
Teflon	±	0	0	0	0
Teflon + Lps	++	++	++	++	+
IL-8					
Collagen	++	++	++	++	++
Teflon	+	±	0	0	±
Teflon + Lps	++	++	++	++	++
IL-10					
Collagen	±	±	±	±	±
Teflon	0	±	±	+	++
Teflon + Lps	+	±	±	±	0

expressing RM3/1 antigens – the glucocorticoid inducible CD 163 scavenger receptor – appear associated with the down-regulatory phase of inflammation and secrete anti-inflammatory proteins [23, 29, 30]. The 25F9 antigen is found on mature macrophages, epitheloid and giant cells in late stages of inflammatory processes [16].

Therefore, our data strongly suggest that the collagen/elastin membrane causes a shift towards macrophage subsets with inflammatory functions. This is primarly based on the finding that macrophages migrating into the membrane expressed only the inflammatory antigens 27E10 and 25F9 but not the anti-inflammatory RM3/1 antigens. Macrophages which did not migrate into the membrane appear to also exhibit an inflammatory phenotype even though the observed increase in 27E10- and decrease in RM3/1-macrophages did not reach statistical significance. The observed decrease in 25F9 macrophages might be due to the invasion of these cells into the membrane. It might also be possible that the specific microenviroment present on or within the membrane differently affected macrophage maturation to the 25F9 phenotype.

The cytokine expression and modulation of macrophage phenotype indicates that the membrane causes inflammation, a key feature for the initiation of wound healing. The lack, however, of ''healing'' RM3/1 macrophages and the predominance of chronic 25F9 cells within the membrane might be an indication for a chronic type of inflammation elicitated by the membrane.

This is supported by the experiments using the CAM-assay demonstrating that the implantation of the collagenous matrix induces the formation of granulation tissue. Moreover, grafting experiments in rats also revealed the appearance of a granulomatous inflammation in which giant cells were detectable corroborating the chronic type of inflammation [15]. Nevertheless the presence of granulocytes seen in the CAM-assay as well as in the animal studies points also to an acute inflammatory reaction [15, 24]. Thus the membrane appears to elicit an inhomogenous inflammatory response.

In the CAM-assay the membrane stimulates angiogenesis, an important aspect in tissue repair [6]. However, neovascularization is also necessary to maintain a chronic inflammation which can be extremely damaging to normal tissue thereby counteracting tissue repair processes [31]. This might be one reason for the scar contraction although being moderate observed in grafting experiments using the collagen/elastin scaffold [15].

5. Conclusions

The data of the present study show that the collagen/elastin membrane meant to be used as dermal substitute causes activation of macrophages by inducing the expression of inflammatory cytokines and the shift in macrophage subsets towards inflammatory phenotypes. Moreover, the dermal scaffold stimulates angiogenesis and the formation of granulation tissue. Although these processes are essential for wound healing, the type of inflammation which resembles at least in part a chronic inflammatory reaction might counteract an efficient wound healing.

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