The chorioallantoic membrane of the chick embryo as a simple model for the study of the angiogenic and inflammatory response to biomaterials

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Angiogenesis is essential in wound healing and a common feature in chronic inflammation which is crucially involved in the biological response to biomaterials. A useful system to evaluate the angiogenic activity and the inflammatory potency of various agents is the chorioallantoic membrane (CAM) of the chick embryo. Here we examined its response to different biomaterials. Smooth materials such as PVC or the polyurethane Tecoflex® either unmodified or modified by an OH- or $N(CH_3)_3^+$-end group (HEMA or MAPTAC) inhibited angiogenesis and did not induce the formation of granulation tissue. The anti-angiogenic effects of PVC, Tecoflex® and its HEMA modification, however, were only seen at an early stage of development. In contrast, the MAPTAC modified Tecoflex® inhibited angiogenesis over the whole time. Rough materials, e.g. filter paper or a collagen/elastin membrane, stimulated angiogenesis and induced the formation of inflammatory tissue. Histological analysis revealed that the filter material was homogeneously populated with cells consisiting mainly of macrophages, fibroblasts and endothelial cells. The collagen/elastin membrane was only partially infiltrated with cells. Among those also clusters of granulocytes were present pointing to an acute inflammatory process. These data show that the angiogenic activity and inflammatory response of biomaterials strongly depend on the chemical composition and the physical structure of the material. The CAM assay appears to be a useful tool for studying biocompatibility.

1. Introduction

The implantation of biomaterials initiates biological responses leading to inflammatory reactions particularly of the chronic type. It has been recognized that the chronification of inflammation is accompanied by angiogenesis [1]. This process, however, is also an important aspect in wound healing particularly in scar formation [2]. While a chronic inflammation represents a typical adverse effect with respect to permanent implants, an efficient scar formation might be of therapeutic relevance, e.g. in skin replacement or in abdominal wall replacement by degradable mesh inplants [3, 4].

The chorioallantoic membrane of the chick embryo is widely used for studying angiogenesis *in vivo* and is referred to as the CAM assay [5, 6]. It is least costly, easier to use and of limited ethical concern than other *in vivo* models. Moreover, it can serve as a model for inflammation [7, 8]. Only limited data, however, is available about the influence of materials, particularly biomaterials, in the CAM assay [9, 10]. Here we employed this classical model to study the qualitative and quantitative effects of chemically and structurally different biomaterials on angiogenesis as well as on the formation of inflammatory tissue.

2. Materials and methods

2.1. CAM assay

Fertilized hen eggs from Bücherhof (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 (Ø 12 mm) of the sterilized test biomaterial was placed on the chorioallantoic membrane. The opening in the shell was sealed with paraffin to prevent dehydration and the eggs were incubated at 37 °C for another 7 or 9 days (angiogenesis) or 8 days (inflammation). In preliminary experiments these incubation periods were found to be optimal for either the investigation of the time course of the neovascularization or the induction of granulation tissue [7]. At the end of these periods the membrane was either fixed *in situ* using 2% buffered formalin, excised and mounted on a slide, or the disk together with the underlying granulation tissue were 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. The average of the numbers of small vessels (less than 20–40 μm) of these areas was taken as vacularization index (see results).

Statistical analysis was carried out using students *t*-test for unpaired samples.

2.2. Materials

The materials tested included the following smooth polymers: the polyvinylchlorides PVC06 and PVC36 containing either the plasticizer diethylhexyl-phthalate or triethylhexyl-trimellitate, respectively (Rehau AG, D) and the polyurethane Tecoflex® as well as Tecoflex® modified by an OH- or $N(CH_3)3^+$-endgroup (HEMA or MAPTAC) using plasma-induced graft copolymerization which was carried out by the Institute of Textile Chemistry and Macromolecular Chemistry of the RWTH Aachen. Furthermore, two rough materials were investigated, e.g. a collagen/elastin membrane (Bioplex Medical BV, NL) and filter paper (Whatman No. 1, Whatman Int. Ltd, Maidstone, GB), the latter serving as a positive control for angiogenesis and the induction of inflammatory tissue. In negative controls no material was implanted.

3. Results

3.1. Angiogenesis

Macroscopical evalution clearly showed a directed growth of vessels towards the collagen or filter discs (Fig. 1a, b). Blood vessels were also present on the materials covering at least half of the disk. Moreover, in about half of the experiments the collagen/elastin

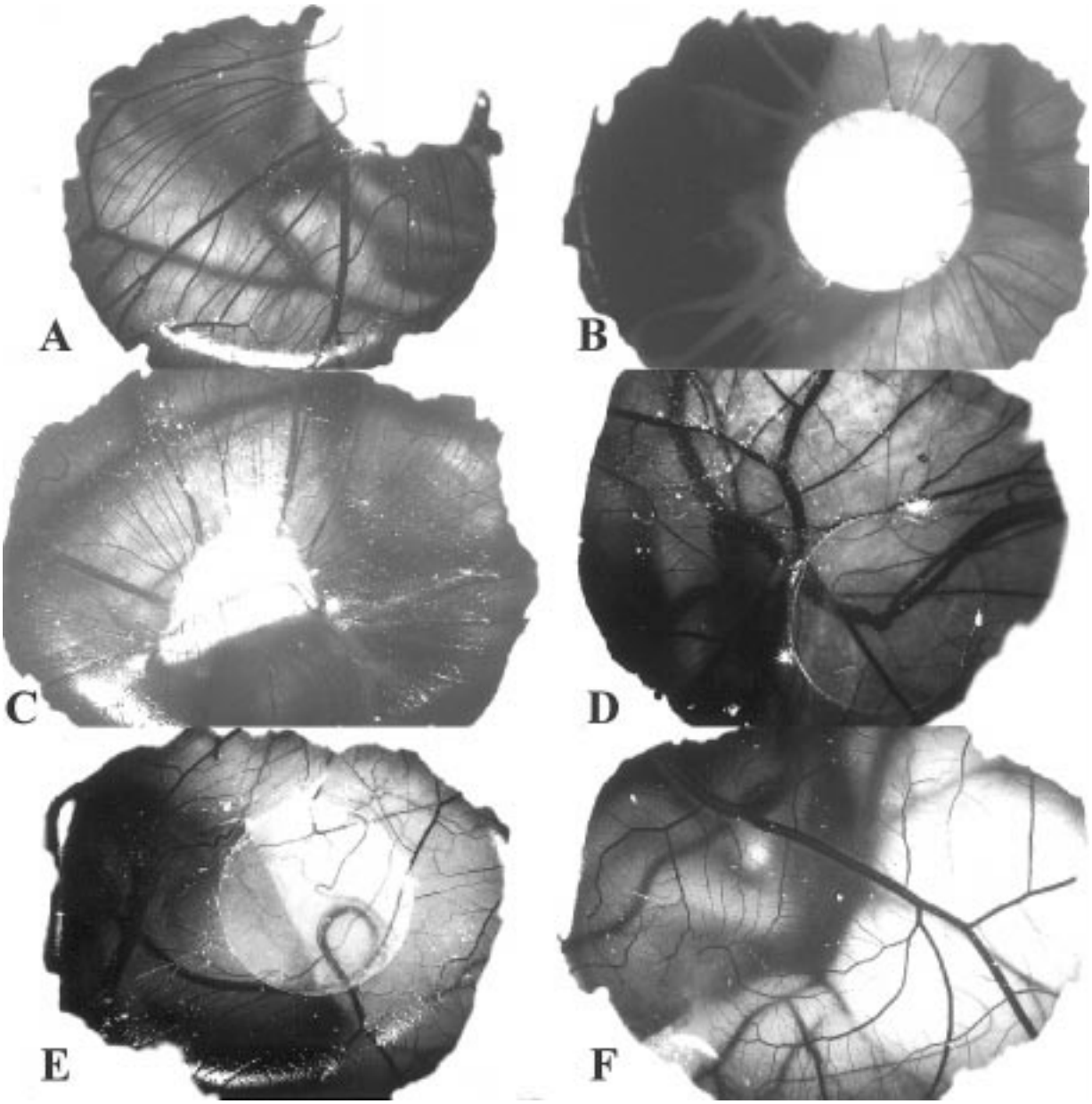

Figure 1 Photographs of the chorioallantoic membrane (CAM) of the chick embryo treated with various materials (12.5 ×). Directed growth of blood vessels to a collagen/elastin membrane (A) and a Whatman filter disc (B) 7 days after implantation. Integration of the collagen/elastin membrane into the CAM after 9 days (C). Randomly distributed blood vessels in the CAM seven days after implantation of PVC36 (D) and nine days after implantation of the Tecoflex® modification MAPTAC (E) and in the untreated control (F).

membrane was found to be completely enclosed by the chorioallantoic membrane (Fig. 1c). The different polymers investigated did not cause a directed growth of blood vessels which were randomly distributed as seen in controls (Fig. 1d–f).

By measuring and counting the thickness and number of vessels it was found that the number of blood vessel smaller than 20–40 μm was significantly ($p < 0.05$) changed by the materials. The thickness as well as the number of vessels with a diameter greater than 40 μm were similarly affected, but a stastistical significance could not be established.

As shown in Table I both, the filter paper and the collagen/elastin membrane stimulated angiogenesis by increasing the number of small vessels/capillaries, the filter paper, however, significantly only after 9 days. In contrast, all polymers were found to inhibit the formation of these capillaries at day 7, but only the MAPTAC modified Tecoflex® suppressed the growth of these small vessels also at day 9.

The anti-angiogenic effects were not due to toxicity of the polymers PVC06, PVC36 and the Tecoflex® modifications HEMA and MAPTAC since the death rates in the embryos treated with the materials were not significantly different from the controls (after 7 days 0–25%, after 9 days 11–32% rates within 14 experiments each including 20–40 eggs). Unmodified Tecoflex® appeared to cause an increased death rate of about 50% although only after 9 days.

3.2. Inflammatory tissue

Histological examination revealed that the collagen/elastin membrane was only partially infiltrated while the filter disk was homogenously populated with cells consisting mainly of macrophages, fibroblasts and endothelial cells (Fig. 2 a–d). In contrast, in the collagen membrane a strong infiltration of granulocytes was found indicating an acute inflammatory process (Fig. 2e, f).

4. Discussion

The CAM assay has widely been used as a simple *in vivo* model to study the angiogenic activity of various agents, e.g. growth factors, cytokines, hormones, drugs, tissue extracts and implanted tissue grafts [5, 11, 12]. Little, however, is known about the reaction of biomaterials in this model although several materials have been used as carrier for test-substances particularly for assaying cell extracts for angiogenic activity [12]. After the initial description of the induction of inflammatory tissue by a sterile filter paper in the CAM assay by D'Arcy & Howard in 1967 [8], Eisenstein and coworkers documented additionally an angiogenic reaction to a Millipore filter membrane [3,4]. In 1978 a systematic study on the angiogenic and inflammatory effects was published using different carrier materials, e.g. glassfiber filters, viscose and gelatine sponges, agarose and polyacrylamide gels [9]. The data indicated that all materials, investigated elicited an inflammatory response but differed with respect to the quantity of the angiogenic induction and the quality of the cellular response.

Based on these early findings here we used the CAM assay to analyze the reaction of materials used or meant to be used as temporary or permanent implants. Our data show that biomaterials strongly differ in their ability to influence the angiogenic and inflammatory response in this *in vivo* model. Materials with a smooth surface such as the PVCs used as catheter materials or the experimental polyurethane Tecoflex® and its modifications appear to be not angiogenic but even anti-angiogenic. Since the inhibitory effects on angiogenesis mediated by the PVCs, Tecoflex® and HEMA-Tecoflex® were only seen at an early stage of development, it seems likely that these polymers exert only a weak and transient influence on angiogenesis. In contrast, the long lasting inhibition on capillary growth of the MAPTAC Tecoflex® points to a clear anti-angiogenic effect. Moreover, these findings strongly suggest that the chemical properties of a polymer might determine the angiogenic response. With respect to the Tecoflex® modifications studied here a positively charged surface appears to be responsible for the anti-angiogenic effect. On the other hand, acid components present in PVC06 and PVC36 by the plasticizer diethylhexyl-phthalate or triethylhexyl-trimellitate, or hydrophilic properties connected with the polyurethane Tecoflex® and its HEMA modification are less inhibitory for capillary growth. The molecular basis of the anti-angiogenic effects remains unclear. It has been suggested that proteins adsorbed to the polymer surface are responsible for the biological response induced by the material [13]. Based on this assumption the chemical structure of a biomaterial clearly determines the type of proteins adsorbed to its surface. In the case of the positively charged MAPTAC a binding of anti-angiogenic molecules with negatively charged groups, e.g.

TABLE I Effect of different biomaterials on angiogenesis measured by the numbers of small (20–40 μm) vessels (capillaries)

Material	Day 7	Day 9
Controls	34 ± 5.5 (24)	36 ± 7.2 (19)
Collagen	40 ± 8.7 (16)*	44 ± 7.0 (12)*
Whatman	37 ± 5.4 (18)	42 ± 7.5 (19)*
PVC06	27 ± 5.8 (9)*	36 ± 6.9 (7)
PVC36	29 ± 2.9 (8)*	39 ± 5.4 (7)
Tecoflex®	24 ± 8.1 (8)*	34 ± 5.5 (3)
HEMA	21 ± 6.9 (8)*	36 ± 6.7 (6)
MAPTAC	23 ± 5.5 (6)*	27 ± 4.2 (6)*

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

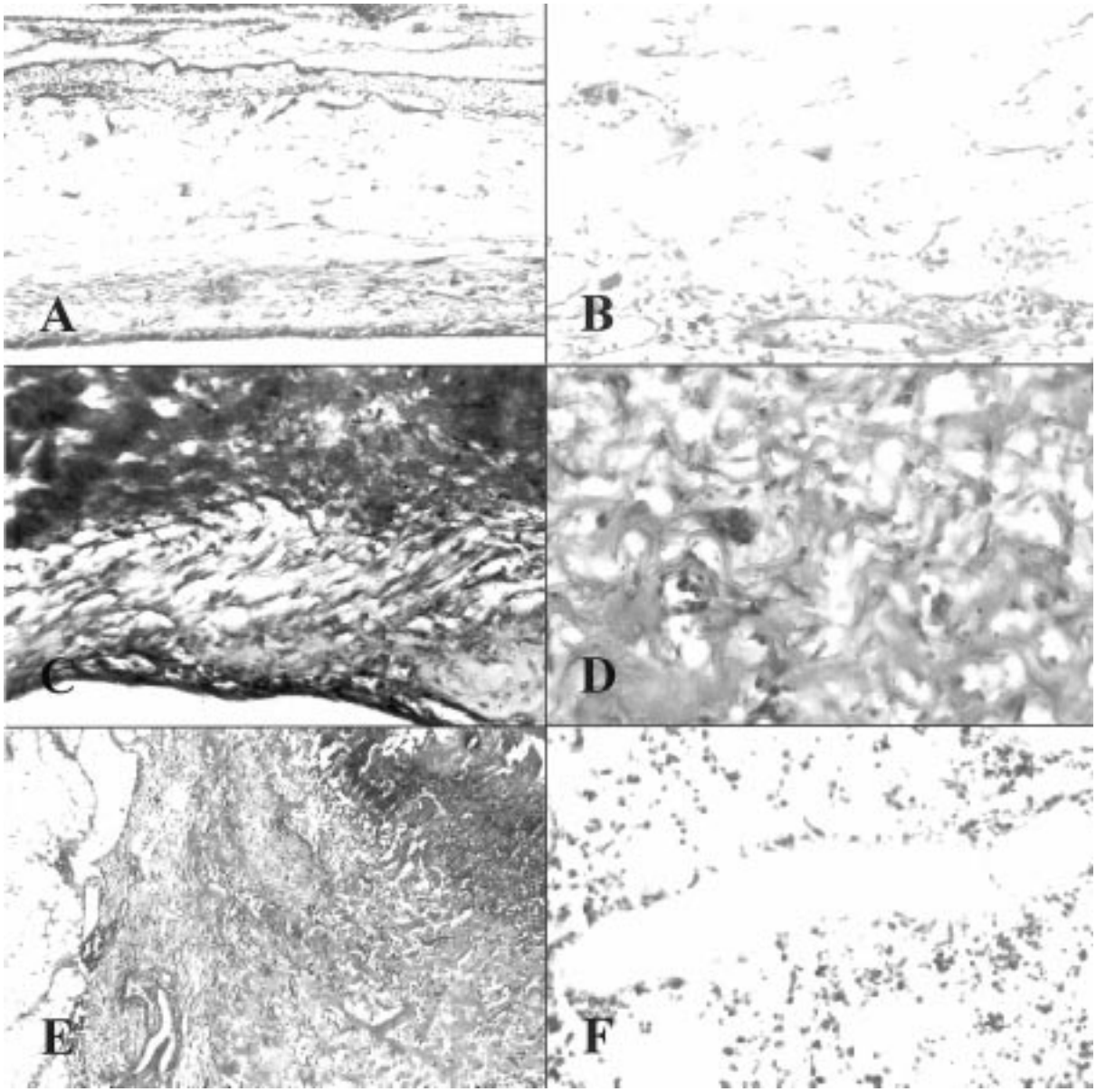

Figure 2 Paraffin sections of the granulation tissue induced by rough materials in the chorioallantoic membrane (CAM) assay (H&E staining). (A) Whatman filter covered on both sides with the CAM and with equally distributed cells within the filter (40 ×). (B) Detail of (A) with macrophages, fibroblasts and proliferating capillaries and missing acute inflammatory reaction (250 ×). (C) Contact area of the collagen/elastin membrane (40 ×). (D) Detail of (C) with proliferating vessels and few macrophages in the membrane (250 ×). (E) Another example of the collagen/elastin membrane with edema and acute inflammatory reaction (40 ×). (F) High power magnification of (E) with a single collagen fiber and a strong granulocyte infiltrate in the interstitium (400 ×).

heparin, might be of relevance but probably also proteins such as angiostatin, a recently discovered endogenous inhibitor of angiogenesis [14].

Angiogenesis seems to be induced particularly by rough materials together with an inflammatory response because the filter paper as well as the collagen/elastin membrane stimulated both, angiogenesis and the formation of granulation tissue. This is in accordance with the early findings by Jacob and coworkers and the general concept of the codependence of angiogenesis and inflammation [9].

The qualitative difference in the cellular reaction to the filter paper used as experimental carrier material and collagen/elastin membrane meant to be used as dermal substitute comprises two features, e.g. the distribution of cellular population and the type of infiltrating cells. The homogenous population of the filter paper suggests a symmetrical architecture of the material. On the other hand, the inhomogenous infiltration of the collagen/elastin membrane with cells might be explained by unevenly distributed elastin fibers within the collagen texture as documented by electron-microscopical investigation [3].

The most striking differences between both materials carrying a probably higher relevance for the biocompatibilty than the cell distribution pattern is the presence of clusters of granulocytes in the collagen/elastin membrane not seen in the filter paper. A high infiltration rate of granulocytes followed by a cover of granulocytes mixed with layers of detritus/necrosis on the surface of the membrane has also been observed in grafting experiments with rats [3]. The persistance of high numbers of granulocytes must be regarded as a clear sign for an acute inflammatory process which might counteract a sufficient wound healing.

In conclusion, our data show that biomaterials strongly

differ in their angiogenic activity and inflammatory response in dependence on the chemical and physical structure of the material. The CAM assay appears to be a useful and simple model to evaluate the angiogenic and inflammatory potency of biomaterials.

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