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Modulation of the tissue reaction to biomaterials

Part I *Biocompatibility of crosslinked dermal sheep collagens after macrophage depletion*

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Although in the last few years in general the biocompatibility of biomaterials has significantly improved, unwanted tissue reactions are often observed resulting in early resorption of the biomaterial, loosening of the implant or in a chronic (immunologic) response. From immunologic studies it is known that inflammatory reactions can be modulated by use of (anti) growth factors or anti-inflammatory drugs. Before this can be employed the role of individual factors (humoral and cellular) involved in the inflammatory reaction against biomaterials has to be studied. In this part of the study the role of macrophages is studied with and without depletion by use of the liposomes-mediated macrophage suicide technique. Crosslinked dermal sheep collagens were used as biodegradable test materials. The results showed that macrophage depletion increases vascularization, and decreases the infiltration of granulocytes into the collagens. The foreign body reaction, i.e. the infiltration of macrophages and giant cells was significantly inhibited, resulting in a strongly delayed degradation time of the biomaterials. However, macrophage depletion did not inhibit attraction of fibroblasts and even resulted in increased formation of autologous rat-collagen, which improved the biocompatibility and the function of the biomaterials as a tempory scaffold.

1. Introduction

In the last few years the application of biomaterials has assumed enormous proportions. Much attention is focused on improvement of biocompatibility by development of new materials, or by modifications of existing materials (e.g. by coating). Degradable and non-degradable materials are used for many kinds of applications, however, unwanted primary and/or secondary tissue reactions are very often observed. This can result in chronic inflammatory reactions, which may cause loosening of prostheses or (early) resorption of biomaterials [1, 2].

Since the development of completely inert materials is often not possible it is desirable to modulate the tissue reactions against biomaterials. Recent studies have shown that tissue reactions which are important for the biocompatibility of biomaterials mainly concern (varieties of) a (sterile) inflammatory reaction. Cellular and humoral factors, such as platelets, granulocytes, macrophages, lymphocytes, fibroblasts, giant cells [2, 3], the coagulation cascade (e.g. bradykinin) [4], complement [5–7], adhesion molecules [8] and cytokines [1, 9, 10] can be involved in the unwanted tissue reaction. However, up to now only a few investigations have [11, 12] reported (e.g. by use of corticoids) the possibility of modulating the tissue reaction, i.e. the sterile inflammatory reaction to biomaterials.

Meanwhile in immunology many studies have revealed possibilities for modulating inflammatory reactions, for example by means of the human interleukin-1-receptor antagonist (IL-1ra) [13], anti-IL-1, anti γ-interferon (anti γIFN), anti IL-6, anti IL-8 [14], tumor necrosis factor (TNF) soluble receptor (TNFsr), or IL-6sr. Furthermore, anti-inflammatory drugs, such as pentoxifylline which blocks TNF-α [15] and DDS (dapsone) which prevents the extravasation of granulocytes, have been used [16].

However, before modulation of the tissue reaction against biomaterials can be performed it is necessary to analyse all individual factors (cellular and humoral) involved in the inflammatory process. This can be done by elimination of the individual factors via several systems: (i) dichloromethylene-diphosphonate (C12MDP) containing liposomes to eliminate macrophages; (ii) "nude" rats, i.e. thymic and T-cell deficient rats; (iii) complement-depleted rats, e.g. by use of the Cobra Venom Factor (CVF); and (iv) Brown Norway rats, which have a kininogen deficiency.

In this first part of the study we investigated the role of macrophages in the inflammatory process as a consequence of applications of biomaterials.

It is known that macrophages, attracted after application, may have different functions such as: (1) release of cytokines, e.g. IL1, IL6, IL8, TNF (see

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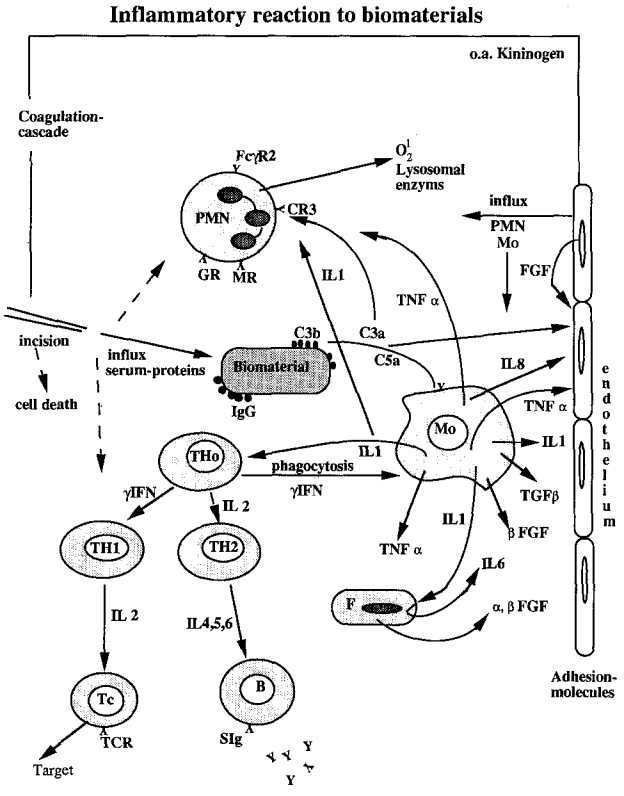

Figure 1 Tentative diagram illustrating the complex cellular and humoral effects after subcutaneous implantation of a biomaterial. PMN = polymorphnuclear cell, C = complement, CR = complement-receptor, GR = glucose-receptor, MR = mannose-receptor, Ig = immuno-globulin, IL = interleukin, Mo = monocyte, TNF = tumor necrosis factor, TGF = transforming growth factor, FGF = fibroblast growth factor, F = fibroblast, TH = T-helper cell, γIFN = γ-interferon, Tc = target cell, S = soluble.

hypothetic diagram, Fig. 1), which in their turn can activate other cells; (2) phagocytosis of cells/materials; and (3) formation of giant cells. Up to now, with respect to these it is not known whether, after elimination, other cell types will take over these functions, or whether the cellular and/or humoral balance will be influenced in a positive way or will be disturbed with respect to the biocompatibility of biomaterials.

2. Materials and methods

2.1. Materials

Liposomes encapsulated drug containing dichloromethylene-diphosphonate (C12MDP), were gratefully obtained from Dr. N. van Rooijen of the Free University of Amsterdam, The Netherlands. The preparation method was previously described in detail by van Rooijen *et al.* [17]. The C12MDP containing liposomes were stored at 4 °C. Before use the drug was centrifuged at 100 g and finally resuspended in 4 ml PBS.

Non-crosslinked dermal sheep collagen (DSC) processed from sheep skin [18], was obtained from the Zuid Nederlandse Zeemlederfabriek, Oosterhout, The Netherlands. DSC was crosslinked either with hexamethylenediisocyanate (HMDIC), to result in HDSC, or with acyl azide (Aa), to result in AaDSC, by Dr J. Feijen at the University of Twente, The Netherlands [19, 20].

For crosslinking with the bifunctional agent HMDIC [20], 1 g samples were immersed in 100 ml 0.07 M NaH_2PO_4 buffer (pH 9.5) containing 1.5% (w/w) HMDIC (z.S., Merck-Schuchardt, Hohenbrunn, FRG) and 1.0% (w/w) Tween 80 (z.S., Merck-Schuchardt, Hohenbrunn, FRG) as a surfactant for 5 h at room temperature. After crosslinking the samples were extensively rinsed to remove unreacted HMDIC or surfactant and lyophilized.

For Aa-crosslinking [21], the carboxylic acid groups of DSC were first ethylated by treating 1 g samples for 6-days with constant mixing in 35 ml ethanol (p.a., E. Merck, Darmstadt, FRG) containing 0.2 M HCl. The samples were then washed with a 0.5 M NaCl-solution and the ethyl ester groups were converted to hydrazides by immersing the samples in 100 ml of a 5.0% (w/w) solution of hydrazine (monohydrate, 99%, Janssen Chimica, Geel, Belgium) in 0.5 M NaCl for 24 h at room temperature. After hydrazide formation, samples were washed with an ice cold 0.5 M NaCl solution. Acyl azide formation was performed in 100 ml of a 0.5 M NaCl solution containing 0.5 M $NaNO_2$ (E. Merck, Darmstadt, FRG) and 0.3 M HCl at 0 °C for 10 min. Washing with 0.02 M Na_2HPO_4 buffer (pH 8.9) containing 0.5 M NaCl was then followed by, finally, crosslinking by reaction of acyl azide groups with amine groups in 200 ml buffer (pH 8.9) containing 0.5 M NaCl at room temperature for 48 h. Thereafter, samples were extensively rinsed and lyophilized.

Discs with a diameter of 8 mm were punched from HDSC and AaDSC (the weight of the discs was approximately 15 mg) and sterilized by ethylene oxide. Previously both materials were stated to be non-cytotoxic and their function as a temporary scaffold was tested [22].

2.2. Methods

NIH guidelines for the care and use of laboratory animals (NIH publication 85-23 Rev. 1985) have been observed. Male 3-month-old PVG rats were ether-anaesthetized. Thereafter 2 ml of C12MDP-liposomes were intravenously injected into the tail of the rats. After 24 h subcutaneous pockets were made to the right and left of two midline incisions on the back. DSC-discs of 8 mm diameter were implanted in the pockets at a distance of about 1 cm from the incisions.

Implants with surrounding tissue were carefully dissectioned from the subcutaneous site after 4, 7, 14 and 25 days.

Immediately after explantation materials were immersion-fixed 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Specimens were cut into small blocks (2 mm^3) after at least 24 h fixation at 4 °C. Blocks were embedded in Technovit 7100 (Kulzer, Friedrichsdorf, FRG) as well as in Epon 812. For both types of embedding, blocks were dehydrated in graded alcohols, but in the case of Epon embedding, blocks were first postfixed in 1% OSO_4 in PBS. Semithin sections (1–3 μ) for light microscopical evaluations were stained with toluidine blue. Ultrathin sections (70 nm) were cut of the Epon embedded materials and

stained with uranyl acetate and lead citrate and examined with a Philips EM 201 transmission electron microscope, operated at 40 kV.

3. Results

HDSC and AaDSC looked similar and a shammy-like appearance with a fibrous and supple structure. At the microscopic level both materials mainly consisted of a matrix of dermal sheep collagen (DSC) bundles.

During explantation, *macro*scopically at day 4 and day 7 strongly increased vascularisation was observed subcutaneously in the liposomes-treated rats (LTRs) as compared with the untreated control rats.

Light-*micro*scopically *at day* 4 with the controls, many granulocytes and some macrophages and giant cells had infiltrated the margins of both HDSC and AaDSC (Fig. 2a). Fibrin fibres formed from wound fluid were present between DSC bundles through almost the entire implant and a thin capsule including small blood vessels had surrounded the implants (Fig. 2a).

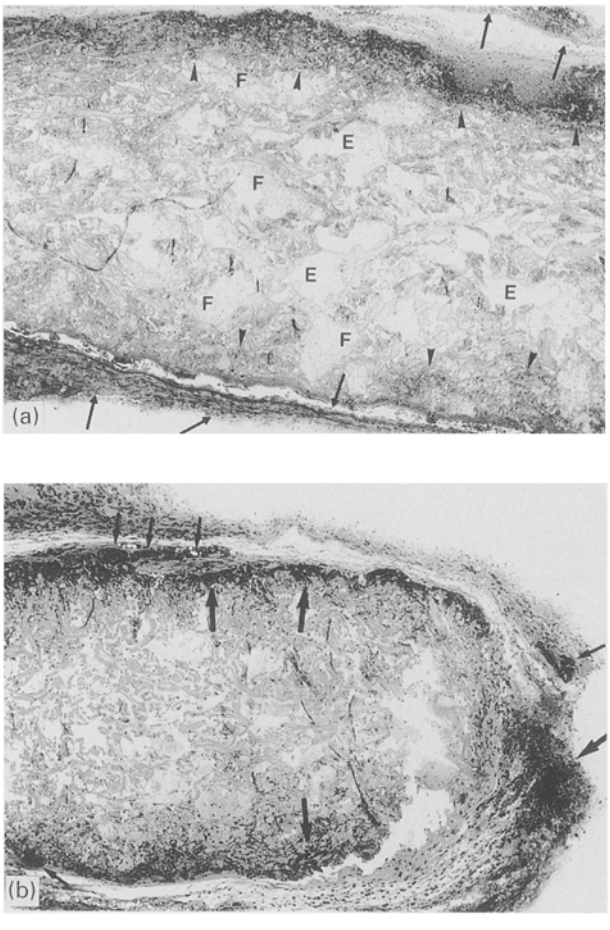

Figure 2 Light micrographic overviews of AaDSC in cross-section 4 days after implantation. (a) The control implant mainly consists of a matrix of larger and smaller collagen bundles with both empty (E) and fibrin (F)-containing spaces. Around the implant a thin fibrous capsule (arrows) with small blood vessels is present. At the margins of the implant some infiltration of granulocytes, macrophages and giant cells (arrowheads) can be observed. (b) The LTR implant shows huge haemorrhages (large arrows), both within the thin fibrous capsule and within the implant. Furthermore many blood vessels (small arrows) were observed. Magnifications: 40x.

In case of LTRs, at this time several granulocytes and sporadically a giant cell had infiltrated both HDSC and AaDSC (Fig. 2b). Beside this, a huge number of erythrocytes, i.e. haemorrhages, which probably resulted from damaging blood vessels during explantation, was observed in the surrounding capsules and the margins of the implants (Fig. 2b). The fibrin network formed in between DSC bundles in the LTRs was comparable with that of the controls.

From day 7 onwards both HDSC and AaDSC in both controls and LTRs, had been completely encapsulated with a fibrous capsule containing many blood vessels (Fig. 3). With the controls $\pm$ 20% of ingrowth had occurred at the margins (Table I). A multi-layer of giant cells and infiltration of some fibroblasts and small blood vessels were observed at the margins of both HDSC and AaDSC. By transmission electron microscopy (TEM) it was shown that giant cells were surrounding, but not yet internalizing, the bundles (Fig. 4a). Granulocytes, which had been present before, were no longer observed.

At the margins of both HDSC and AaDSC of the LTRs only one layer of giant cells was observed (Fig. 3). Small blood vessels and fibroblasts had proceeded deeper than the giant cells (Fig. 3b), but ingrowth in general was $\pm$ 10% less than with the controls (Table I). The fibroblasts were often just aligned with the DSC bundles (Fig. 4b). Furthermore erythrocytes were still loosely present in the implants (Fig. 4b). Granulocytes were not observed.

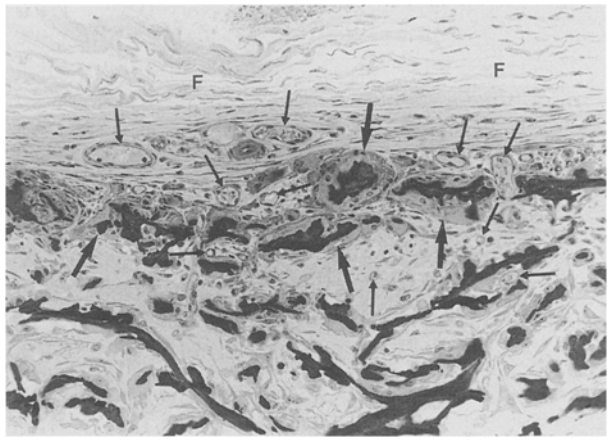

Figure 3 Light micrograph at the margin of the HDSC implant of the LTR at day 7 showing a fibrous capsule (F) with blood vessels (small arrows). Small blood vessels are also present in the implant (small arrows). Furthermore one giant cell-layer was present and surrounded collagen bundles (large arrows). Magnification: 160x.

TABLE I Cellular ingrowth of AaDSC and HDSC

	Controls		Liposome-treated rats	
	HDSC	AaDSC	HDS	AaDSC
Day 4	10%	5%	1–3% [a]	1–3% [a]
Day 7	20%	20%	10% [a]	10% [a]
Day 14	70%	60%	20%	25%
Day 25	100%	90%	90%	70%

[a] Except erythrocyte infiltration.

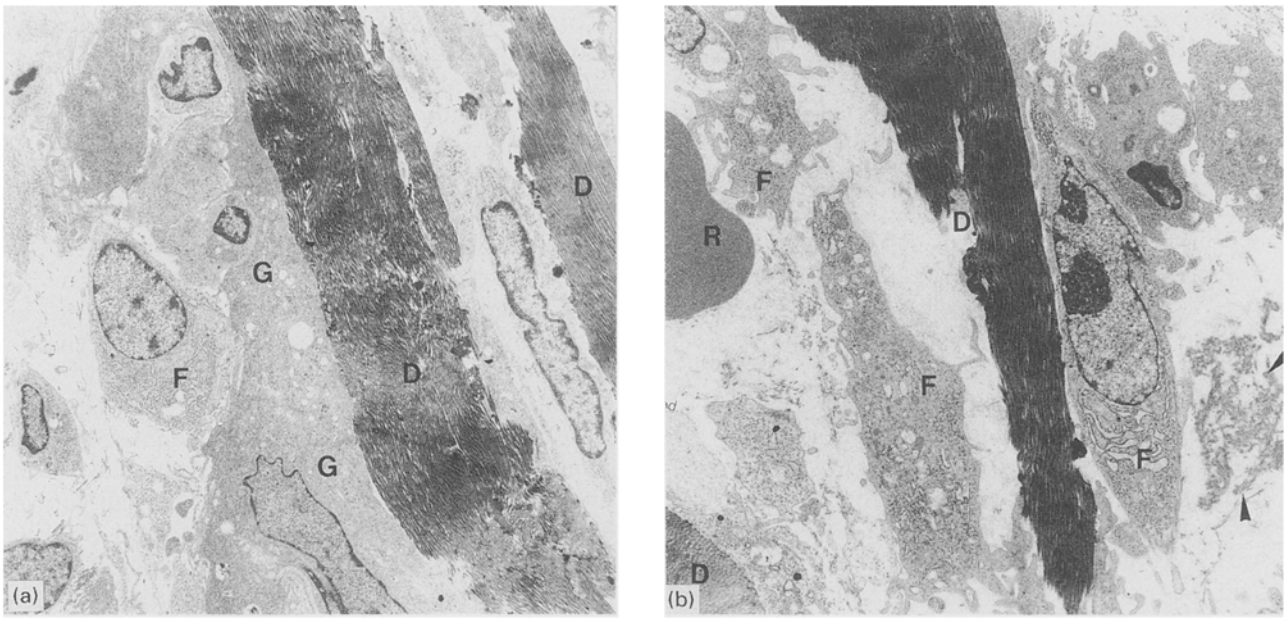

Figure 4 Transmission electron micrographs of AaDSC 7 days after implantation. (a) Control implant with two DSC bundles (D) surrounded by giant cells (G). Furthermore a fibroblast (F) is shown. Magnification:7142x. (b) The LTR implant shows fibroblasts (F) aligning the DSC bundles (D). Furthermore some fibrin (arrowhead) and part of an erythrocyte (R) are observed. Magnification: 4685x.

At day 14 large differences in ingrowth were observed between the controls and the LTRs. Within one group small differences were even observed between HDSC and AaDSC (Table I). With HDSC of the controls, about 70% of the implant had been infiltrated by many giant cells, and some macrophages, fibroblasts and blood vessels. With AaDSC, at this time about 60% was infiltrated (Table I and Fig. 5a).

In sharp contrast, LTRs showed a cellular infiltration of only 20% in the case of HDSC and 25% with AaDSC (Table I and Fig. 5b). TEM revealed that infiltration of AaDSC consisted of many fibroblasts with well-developed rough endoplasmatic reticulum and some giant cells surrounding DSC-bundles; this was even more pronounced with HDSC.

At 25 days the HDSC implant in the controls had been completely infiltrated and DSC bundles had partially disappeared (Fig. 6a). Using TEM, an ongoing degradation, i.e. internalization of the remaining DSC bundles by giant cells, was observed (Fig. 8a). At this time AaDSC showed an infiltration of about 90% (Table I) with, in comparison to HDSC, higher numbbers of fibroblasts and increased formation of collagen (Table II) which were often found in both smaller and larger areas (Fig. 7a). Giant cells had not, as significantly as with HDSC, proceeded to degrade AaDSC, which indicates that HDSC is easier to phagocytose. This agrees with the fact that previously HDSC was found to have a shorter degradation time than AaDSC [22].

In contrast with the group of LTRs, HDSC and AaDSC implants showed ingrowth percentages of $\pm$ 90% and 70%, respectively (Figs. 6b, 7b). Now with both DSCs, besides giant cells also many fibroblasts and the formation of increased quantities of rat-collagen were observed in comparison with the con-

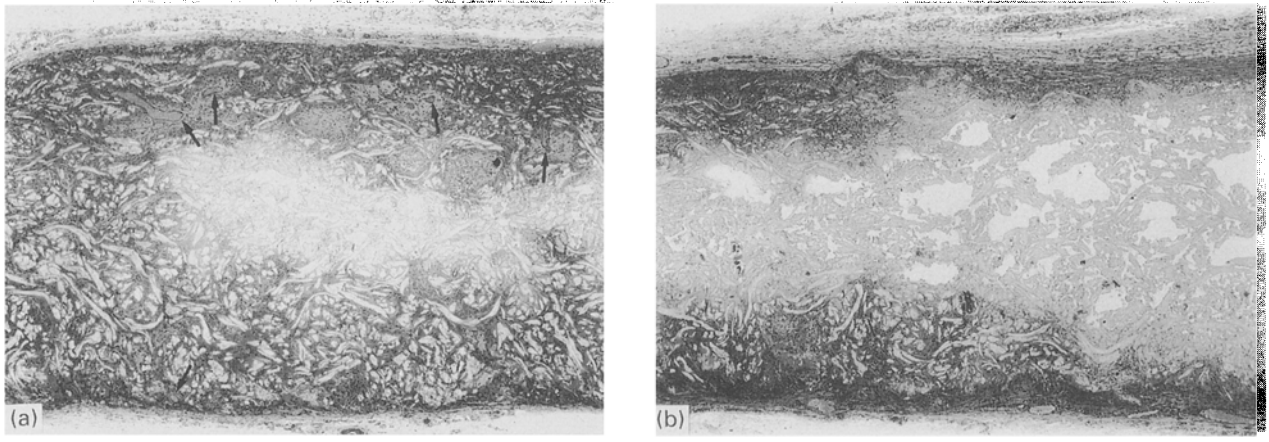

Figure 5 Light micrographic overviews of AaDSC in cross-section 14 days after implantation. (a) The control implant shows $\pm$ 60% ingrowth of DSC with small blood vessels (arrows). (b) The LTR implant shows an inhibited ingrowth of $\pm$ 25%. Magnifications: 40x.

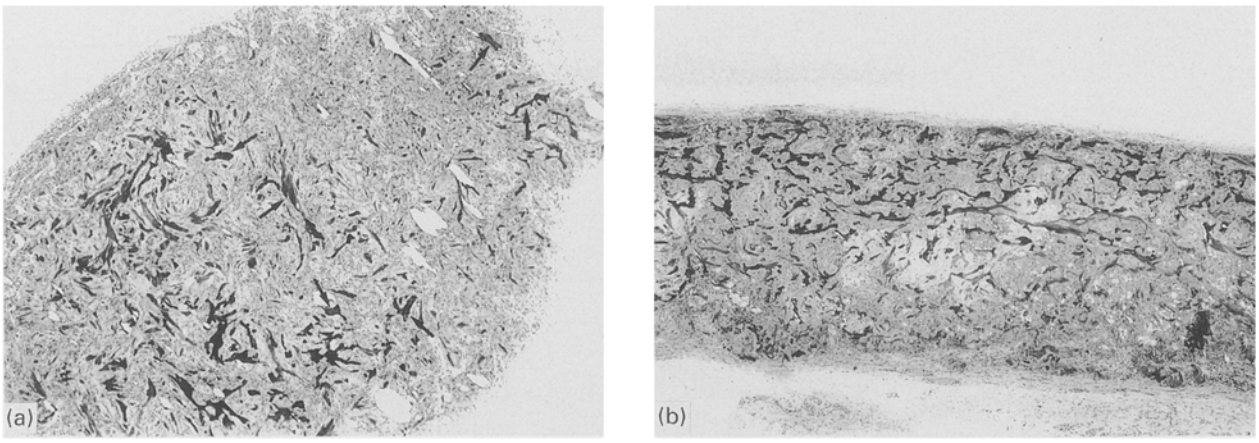

Figure 6 Light micrographs of HDSC 25 days after implantation. (a) The control implant shows complete ingrowth. DSC bundles (arrows) have decreased in size and, in the right half of the picture, have disappeared due to degradation. (b) The LTR implant shows ± 90% cellular ingrowth. Magnifications: 40x.

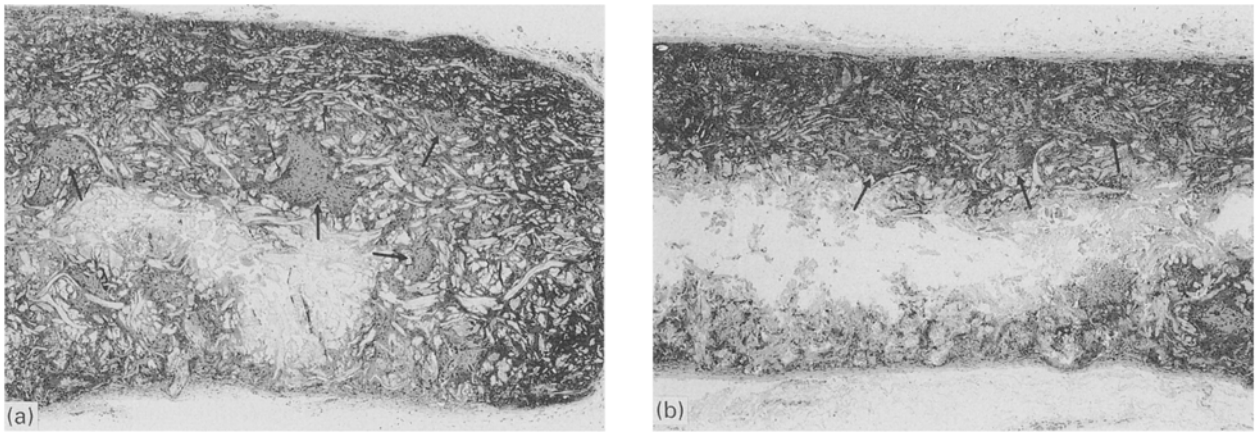

Figure 7 Light micrographs of AaDSC 25 days after implantation. (a) The control implant shows ± 90% ingrowth. Furthermore some rat-collagen areas (arrows) with fibroblasts are present. (b) The LTR implant shows inhibited, i.e. ± 70%, ingrowth. Some rat-collagen areas (arrows) with fibroblasts are present. Artificially the non-infiltrated part of the implant was partially eliminated by specimen cutting. Magnifications: 40x.

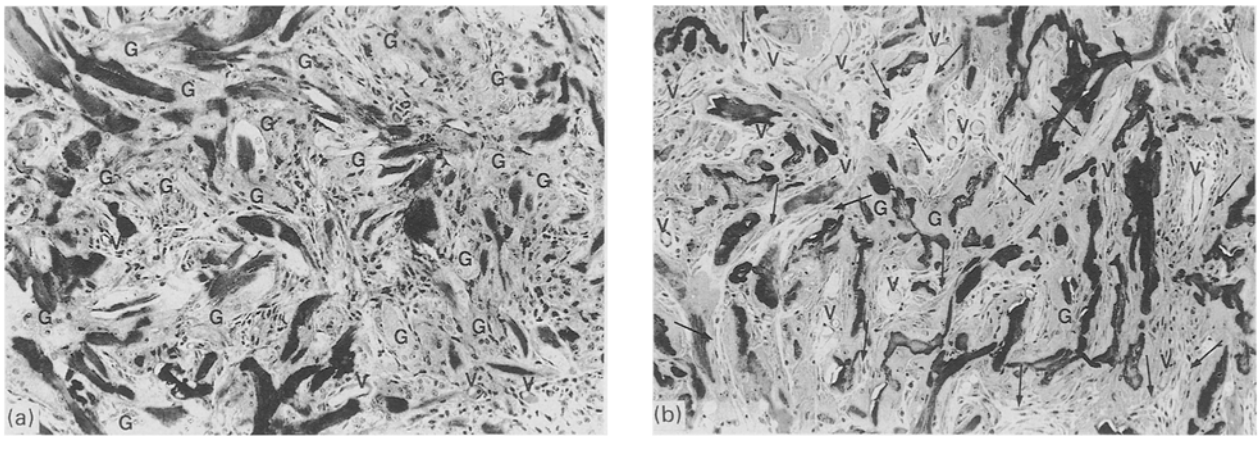

Figure 8 Light micrographs of HDSC 25 days after implantation. (a) Detail of the control implant showing the huge infiltration of giant cells (G), which surround collagen bundles, and a few blood vessels (V). (b) Detail of the LTR implant showing infiltration of fibroblasts and the newly formed collagen (arrows), giant cells (G) and many blood vessels (V) in between the DSC bundles. Magnifications: 160x.

trols. With AaDSC this was formed in many small, but limited, areas, while with HDSC it was not found in limited areas, but was formed in smaller and wider bundles through the entire implant (Fig. 8b). Observations by TEM confirmed that the quantity of newly formed (rat) collagen was clearly increased when comparing the LTRs with the control group, and also, but to a lesser degree, when comparing HDSC with

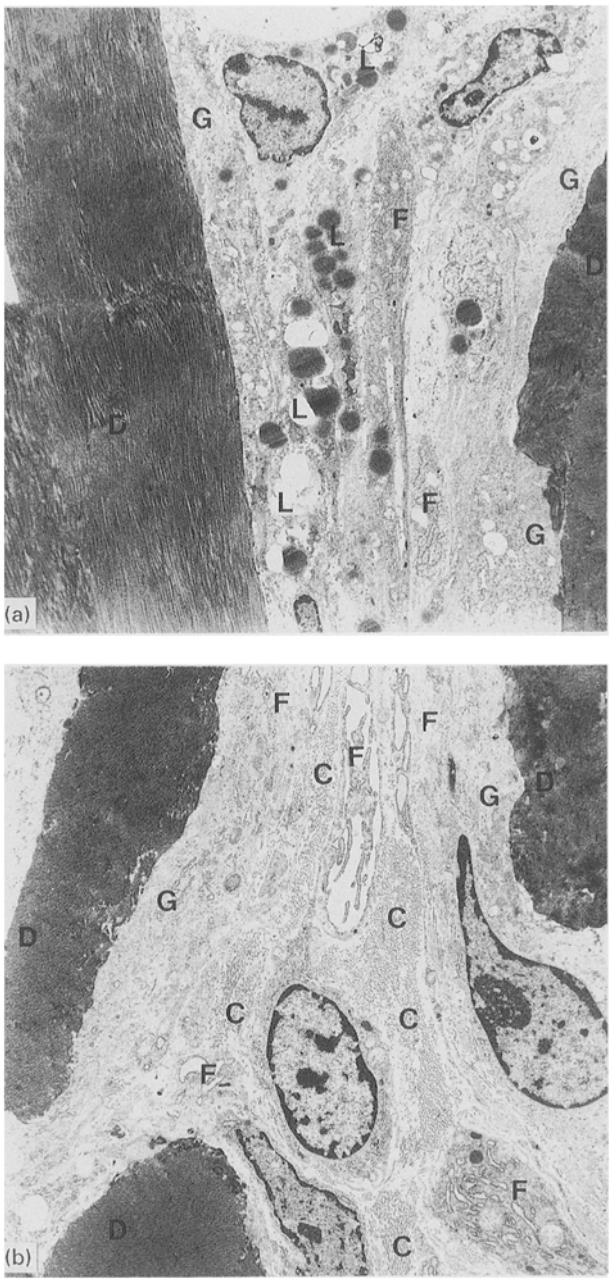

Figure 9 Transmission electron micrographs of HDSC 25 days after implantation. (a) The control implant shows cellular infiltration in between the DSC bundles (D), representing fibroblasts (F), part of giant cells (G) and undefined cells with lipid accumulations (L). Magnification: 4685x. (b) The LTR implant showing increased presence of newly formed rat-collagen (C) and fibroblasts (F) with well-developed rough endoplasmatic reticulum in between DSC bundles (D), which are surrounded by giant cells (G). Magnification: 7142x.

AaDSC (Fig. 9a, 9b). Furthermore, in comparison to the controls, the DSC bundles of the LTRs were less degraded and a higher number of blood vessels was observed in between these bundles and the newly formed collagen.

4. Discussion

C12MDP liposomes ingested by macrophages fuse with primary lysosomes in which the phospholipid bilayers are disrupted by phospholipases, leading to release of toxic C12MDP. By this model macrophages can be eliminated in peritoneum, spleen, liver, lung and lymphnode [22] (as indicated by cell numbers, ecto-enzyme patterns and surface markers of macrophages), but probably not in the dermis. A single administration of C12MDP-liposomes was found to eliminate splenic macrophages within 24 h and reappearance did not begin until after 1 week [23]. This was the reason why our group chose to inject liposomes 24 h before application of the biomaterials.

TABLE II Tissue ingrowth of DSCs at day 25

	Controls		LTRs	
	HDSC	AaDSC	HDSC	AaDSC
Giants	+ + +	+ + ±	+ +	+ +
Fibroblasts	+	+ +	+ + +	+ + +
Rt-collagen	+	+ + ±	+ + + +	+ + +
Blood vessels	+	+ +	+ + +	+ + +

Results with respect to (1) infiltration of macrophages and giant cells, (2) angiogenesis, (3) infiltration of granulocytes, and (4) fibroblast ingrowth and formation of collagen, are discussed below.

(1) Our results show that infiltration of macrophages and giant cells is clearly inhibited in the implanted collagens of the liposome treated rats (LTRs) up to 25 days (Table 1). Reappearance began slowly after 1 week, which agrees with reported splenic macrophage recovery [22]. This indicates that infiltration of macrophages and probably also of giant cells into biomaterials must be related to circulating macrophages.

(2) Macrophages, as judged by activation of these cells [24, 25], were found to be important in the early phase of wound healing, i.e. for debridement, fibroblast activity and angiogenesis. However, our results show that down-regulation of circulating macrophages at first strongly increased vascularization, as macroscopically observed at the subcutaneous site and microscopically in the implant, for up to 7 days.

According to Albelda [8] after tissue damage in general, as a consequence of injury, platelets will bind (e.g. via integrins) to subendothelium and the coagulation cascade will generate platelet aggregation. Thereafter an inflammatory reaction is initiated by mediators from platelet granules, followed by endothelial and white blood cell activation and extravasation of leukocytes. Now, activated platelets and white cells will accumulate at the injury site which in their turn up-regulate adhesion receptors on fibroblasts, macrophages and epithelial cells. This model shows that macrophages in the first instance are not required for endothelial activation. Furthermore, in the early phase of healing, fibroblasts will migrate to the injury site and are in this case also attracted to encapsulate and infiltrate the implanted biomaterial. With all these cellular actions cytokines are known to be very important for intercellular communications. Root *et al.* [26] reported that fibroblasts can express multiple acidic and basic fibroblast growth factors (αFGF and

βFGF), while others have demonstrated release of FGFs by endothelial cells [27, 28] and release of βFGF by macrophages [27, 29]. Furthermore FGFs were found to be potent inducers of metalloproteinases [30] and of angiogenesis *in vitro* and *in vivo* [31–33]. With respect to angiogenesis, Passaniti *et al* [31] reported IL 1b, IL6, and TGFβ, all cytokines largely originating from the macrophage lineage, to be potent inhibitors of the angiogenetic response. These combined reports may explain the huge vascularization observed up to 7 days with the LTRs, because insufficient inhibitors IL 1b, IL6, and TGFβ may have been released at that time, while the angiogenesis-stimulators FGFa and FGFb were probably abundantly present, as observed by the presence of many fibroblasts and endothelial cells. Moreover, as hypothesized in Fig. 1, activation of endothelial cells may also occur by the coagulation cascade and complement activation: their roles in the application of biomaterials will be discussed in a follow-up paper.

(3) Compared to cytotoxic materials (21), DSCs in control rats attracted low numbers of granulocytes. However, these numbers were still significantly higher than the numbers attracted by DSCs in the LTRs. Derijk *et al.* [34] concluded that circulating IL1 concentrations in rats, largely originating from the macrophage lineage, play a signalling role in the so-called acute-phase response, e.g. by activating/attracting granulocytes and lymphocytes. This may explain the reduced inflammatory reaction of HDSC in the LTRs. Influences of immunoglobulins and/or complement, which can, as shown in Fig. 1, also activate granulocytes, will be discussed later. However, this study showed that the presence of macrophages is essential for the attraction of granulocytes but, as a consequence of the absence or blocking of specific macrophage-related cytokines, can inhibit the acute inflammatory reaction in response to biomaterial application.

(4) With respect to the fibroblasts, several investigators have demonstrated the beneficial effect of macrophages to fibroblast activity and subsequent collagen synthesis [24, 35]. According to this, after macrophage depletion a decrease of fibroblast activity (such as proliferation, synthesis of metalloproteinases and synthesis of collagen) might be expected. However encapsulation of DSCs, both in the control and LTR group, with fibroblasts and formation of rat-collagen was clearly observed after 4 days, which implies that many fibroblasts were also attracted and activated in LTRs. From these findings we can conclude that macrophages are not indispensable for attraction and activation of fibroblasts as hypothesized in Fig. 1. Fibroblast activity in the LTRs might be initiated by platelet-related factors, autocrine function of fibroblasts, or other white cells and endothelial cells.

Furthermore, from day 7 onwards fibroblast ingrowth and the formation of rat-collagen was significantly increased with DSCs of the LTRs as compared to the controls. Because macrophage depletion, as stated above, delayed infiltration of macrophages and giant cells and thus the degradation rate of DSC, fibroblasts can with less competition infiltrate the DSCs thereby improving their temporary scaffold function. At day 25 this was more predominant with HDSC for two reasons: (1) HDSC was found to be degraded prematurely by giant cells, while AaDSC was not [21]. This was also related to the shrinkage temperature for HDSC and AaDSC (74 °C and 84 °C, respectively) and the differences in sensitivity for degradation by collagenase *in vitro* [19, 20]; (2) because it concerned absolute numbers of fibroblasts/collagen and AaDSC in the LTR group was not completely infiltrated at 25 days. However, previously in the long-run AaDSC was found to show improved function as a temporary scaffold for ingrowth of autologous collagen [21], which also may indicate that it will perform even better in the long-run after macrophage-depletion.

5. Conclusions

This study showed the specific role of macrophages and the complex role of cytokines with respect to applications of biomaterials.

Macrophages play an essential role in regulating angiogenesis and the infiltration of granulocytes and giant cells, but do not have an indispensable role in infiltration of fibroblasts.

Furthermore, the decrease of macrophages, giant cells and granulocyte infiltration, and improvement of the recovery of original tissue give promising possibilities to modulate the tissue reaction against biomaterials. Since the use of the macrophage suicide technique might not be attractive, specific macrophage modulation by the use of immunomodulators such as IL-1ra, as a.o. investigated by Thompson *et al.* [13] and discussed with respect to medical practice by Van der Meer *et al.* [36] and Takx-Kohlen [37], or by the use of proteins against cytokines, or soluble receptors, might be promising alternatives.

However, first, as discussed above, further experiments on the relevance of other factors as hypothesized in Fig. 1 have to be performed in order to completely understand and to be able to fine tune modulation of the inflammatory reactions against biomaterials to improve their biocompatibility and to avoid failures.

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