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Research paper

Pharmacological modulation of the tissue response to implanted polylactic-co-glycolic acid microspheres

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Abstract

Polylactide microspheres have been identified as a promising release system for the sustained delivery of protein therapeutics. We prepared microspheres (30–50 microns) from either polylactic acid monomers (PLA), polylactic-co-glycolic acid (PLGA) monomers containing 12-carbon end groups (blocked PLGA, B-PLGA) or unmodified polylactic-co-glycolic acid monomers (unblocked PLGA, UB-PLGA) and injected these subcutaneously into the interscapular region of rats. Striking differences were observed in the cellular and fibrotic responses to these polymers monitored at various times over a 30 day time course. Incorporation of recombinant human insulin-like growth factor-I (rhIGF-I) into B- and UB-PLGA microspheres at a loading of 14.8 and 15.5%, respectively, did not alter the tissue response to these polymers. Infusion of various agents intended to pharmacologically modify cellular and fibrotic events associated with the tissue response demonstrated that such a manipulation was possible. Together our results demonstrate that the inherent physical and chemical properties of PLA, B-PLGA and UB-PLGA dictate biological aspects of the tissue response to a large extent but open the possibility of modulating these tissue response events through pharmacological intervention. © 1997 Elsevier Science B.V.

Keywords: Biocompatibility; Inflammation; Microspheres; Polylactic acid; Polylactic-co-glycolic acid; Protein delivery; Tissue response; Wound healing; Sustained release

1. Introduction

The tissue response involves a complicated series of biological events. This response is a continuum of interactive and redundant processes [1] involving neural, vascular, cellular and humoral responses [2]. Some of these responses can be protective, such as phagocytosis of invading pathogens, as well as potentially injurious, as in the case of the release of oxygen-derived free radicals [1]. Vascular and cellular components of inflammation are directed by mediators which evoke vasodilatation and increased vascular permeability, as well as chemotaxis of leukocytes and fibroblasts. The body mounts a response not only to eliminate or wall-off the implanted material, but also to repair the tissue injury resulting from the implantation event itself [3].

Previous work by Anderson and colleagues [4] has described cellular and non-cellular events associated with the tissue response to implanted foreign materials (Fig. 1). These biological events can be artificially partitioned into separate stages. Early events involve acute and chronic inflammation followed by an intermediate stage of tissue granulation. Finally, the area of injury or implantation shows resolution of tissue trauma with a return to the original cellular composition and remodeling of the scar structure [4]. The distinct cellular events associated with each stage and the degree of intensity and duration of each depends on the biocompatibility of the foreign material [5]. Decreasing the intensity or duration of, or outright ablating, aspects of the re-

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sponse improves the body's tolerance to a given foreign material, which in turn may either enhance or diminish the functionality of the implanted device. A number of factors which contribute to the biocompatibility of implanted foreign materials have been well described in the literature [4–8]. Previous investigators have shown the tissue response to be influenced by the shape, size, physical and chemical properties of the implanted material [8–10].

Polylactic-co-glycolic acid (PLGA) microspheres have been shown to be biocompatible [11] and have been investigated extensively for use as protein delivery systems [12]. The polymer composition (ratio of lactide to glycolide) can be modified such that protein can be released over a period ranging from days to more than a year [13,14]. Polylactides have been used for several years as a depot formulation with LHRH agonists and in resorbable sutures for over 20 years without adverse effects [12]. Conversely, previous investigators have suggested that polylactic acid (PLA) or PLGA microspheres might act as adjuvants [15,16] and explored the use of polylactides in vaccine development. What properties the microspheres possess that confer this capability is not clear; however, the continual presence of the microspheres as well as the delivery of an heterologous protein may result in an immune response by design. In general, the polylactides are well-tolerated, but each formulation must be considered independently as several factors would contribute to the potential tissue response to the material. Indeed, alterations in the physical and chemical characteristics greatly affect the potential therapeutic value of an implanted material by either producing no adverse reaction or precipitating a rejection of the device [9].

In the following studies we examined the biocompatibility of three types of placebo polylactide microspheres implanted subcutaneously in a rat model and evaluated the tissue response at these injection sites.

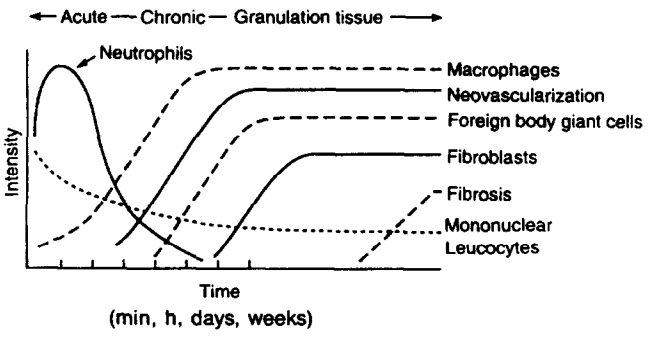

Fig. 1. Temporal nature of cellular and fibrotic events associated with the tissue response. The Y-axis depicts relative intensity or magnitude of the various cell types or tissue events associated with the tissue response. Sequence of each tissue response-associated event can take place over a time-span lasting from days to weeks. The figure was modified from Anderson [9].

Additionally, polylactide microspheres loaded with recombinant human insulin-like growth factor (rhIGF-I) were similarly evaluated. The rat model was modified slightly to deliver one of a variety biologically-active agents to the injection site using Alzet® minipumps in an attempt to pharmacologically manipulate various aspects of the tissue response. These agents included those secreted by activated macrophages or those known to affect inflammation or the immune system. Our findings show clear differences in the tissue response at depot sites for UB-PLGA, B-PLGA and PLA microspheres. Although cellular and fibrous events in the tissue response to these implanted materials could be modified through pharmacological manipulation, the choice of polymer, as well as its form, may be the prime determinant of the biocompatibility for a polymeric drug delivery system.

2. Experimental

2.1. Materials

Cyclosporin A (Csp-A)(Sandimmune®) was obtained from Sandoz (East Hanover, NJ). Levamisole (Leva), dexamethasone-water soluble, sodium heparin monosulfate (average molecular weight: ≈ 7500) and recombinant human basic fibroblast growth factor (rhbFGF) expressed in *E. coli* were from Sigma (St. Louis, MO). Castor oil was from Brite-Life (Orange, CA). Murine tumor necrosis factor-α (rmTNF-α) was produced in *E. coli* [17]. Human insulin-like growth factor-I (rhIGF-I) and human transforming growth factor-β1 (rhTGF-β1) were produced and purified at Genentech, Inc. (South San Francisco, CA) using recombinant expression technologies. The A and B chains of human relaxin (hRlx-2) were chemically synthesized by solid-phase methods and chomatographically purified to a purity of $\geq 98\%$ [18].

2.2. Microsphere preparation

Poly-D,L-lactic-co-glycolic acid microspheres were made from two different polymers: blocked, 12 kDa, 50:50 L/G (0.2 dl/g; RG502) and unblocked, 12 kDa, 50:50 L/G (0.2 dl/g; RG502H) obtained from Boehringer (Ingelheim, Germany). The blocked polymer (B-PLGA) contained hydrophobic end groups (C12) and the unblocked polymer (UB-PLGA) had hydrophilic end groups [19]. Poly-D,L-lactic acid (PLA) microspheres were prepared from polymer (0.22 dl/g) obtained from Medisorb Technologies (Cincinnati, OH). Briefly, a water-in-oil emulsion was produced by dissolving either PLA or PLGA in ethyl acetate and homogenizing this solution with buffer or a concentrated solution of recombinant human insulin-like

Table 1
Agents infused for 14 days into microsphere injection sites

Test agent[a]	Molecular weight (Daltons)	Dose	Anticipated effect
Csp-A	1201	140 mg/kg	Immuno-suppressive
Dex	392	0.8 mg/kg	Anti-inflammatory
rhbFGF	17 000	250 ng/kh	Neovascularization
Leva	204	60 mg/kg	Immuno-potentiator
hRlx-2	5982	0.67 mg/kg	↓Collagen content
rhTGF-β1	12 786[b]	10 μg/kg	Immuno-modulation
	25 572[c]		↑Collagen synthesis
rmTNF-α	17 350[b]	0.9 mg/kg	↑Inflammation
	52 050[d]		↓Collagen synthesis

[a] Agents and methods of infusion are described further in Section 2.
[b] Monomer, [c] Covalent homo-dimer, [d] Compact homo-trimer.

growth factor-I (rhIGF-I). Water-in-oil emulsions were mixed with 9% polyvinyl alcohol and 10% ethyl acetate to form microspheres which were hardened by dilution into 12 l of water. Microspheres were washed with 30 l of water in a 1.5 l Amicon stirred cell with a 20 μm mesh and filtered through a 150 μm mesh while wet.

2.3. Microsphere characterization

After air drying and dry sieving to remove large aggregates (85 μm mesh), particles were sized using a Brinkman Particle Size Analyzer Model 2010 (Lens A, 1–150 mm range). Surface morphology was assessed by scanning electron microscopy of dried microspheres which had been sputter-coated with 10 nm gold-palladium using a Hummer XP coating chamber (Anatech). Protein loading was determined by optical absorbance ($E_{276} = 0.645$ ml/mg·cm) following dissolution of the microspheres in 1 N NaOH. Microspheres were injected in a suspension composed of 3% carboxyl methyl cellulose and 0.1% Tween-20 in phosphate-buffered saline.

2.4. Test agents

Test agents designed to pharmacologically modify the tissue response were co-administered to only the microsphere implantation sites of the IGF-loaded PLGA microspheres. Alzet® minipumps (Model 2002, Alza Corp. Palo Alto, CA) were fitted with a 2 mm internal diameter polyethylene catheter, filled with test agents listed in Table 1, and primed in normal saline at ambient temperature for 12 h prior to implantation. Test agents were prepared in normal saline for injection. A four-fold molar excess of heparin sulfate (0.6 μg/ml) was included in pumps delivering rhbFGF, to saturate the heparin-binding sites on the bFGF, to minimize its association with heparin-like sites in the extracellular matrix. Csp-A was used as prepared (in castor oil) and a control group of animals receiving castor oil was included. All minipumps delivered test agents to the injection site for a 2 week duration at approximately 0.5 μl/h.

2.5. Animal model

Male Sprague-Dawley rats (200–250 g) were anesthetized with ketamine-xylazine (62.5 and 12.5 mg/kg, respectively) and prepared for aseptic surgery. A 1–1.5 cm incision was made along the dorsal midline 3–4 cm caudal to the interscapular region. A narrow tunnel was made beneath the panniculus carnosus in a lateral and caudal direction by blunt dissection and primed minipumps with attached catheters were inserted caudally in the tunnel with the blunt end first such that the catheter end opening was proximal to the interscapular region, the intended microsphere injection site (Fig. 2).

Fig. 2. Approximate relationship of microsphere depot site and catheter tip delivering test agents in the interscapular region of Sprague-Dawley rats. Site of minipump insertion is noted by two surgical clips used to close wound.

The incision was closed with two or three metal surgical clips. The catheter end was palpated and 0.2 ml of the microsphere formulation was injected in the interscapular region, approximately 1 cm from the catheter tip. All animal protocols adhered to the Principles of Laboratory Animal Care (NIH publication # 85–23, revised 1985).

2.6. Histology

Two animals from each group were sacrificed by carbon dioxide asphyxiation on 3, 7, 10, 17, 21 and 30 days following dosing. Microsphere masses and associated fascia were recovered, fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned (4 microns thick) and mounted on glass slides. Mounted sections were stained with either hematoxylin and eosin (HE) or Masson's trichrome (TC) to enhance visualization of cellular or collagen components, respectively.

3. Results and discussion

3.1. Microsphere characterization

We prepared and evaluated the tissue response to five different preparations of polylactide microspheres. Three preparations were composed solely of polymer: UB-PLGA, B-PLGA and PLA. Two preparations were loaded with a therapeutic protein, insulin-like growth factor-I: IGF-I loaded UB-PLGA (IGF-I/UB-PLGA) and IGF-I loaded B-PLGA (IGF-I/B-PLGA). The morphologies of the polymer-only B- and UB-PLGA were more disparate, while the appearance of the implanted PLA had morphological features of both PLGA microspheres. For the protein-loaded microsphere study we therefore chose to study the two polymer types which showed two different morphologies, and thus no protein-loaded PLA microspheres were studied. All five microsphere preparations contained spherical structures having mean diameters consistent with the 30–50 micron values measured by light scattering studies (data not shown). Particle size distributions and surface characteristics were comparable for all five preparations, as observed by scanning electron microscopy (Fig. 3a–e).

3.2. Tissue response to UB-PLGA, PLA and B-PLGA microspheres

Cellular and fibrotic events of the tissue response to UB-PLGA, PLA and B-PLGA microspheres were followed for up to 30 days following subcutaneous (SC) injection into the interscapular region of rats. Histological preparation of excised SC injection sites extracted the polylactide polymer, enabling improved examination of the cellular infiltrate and the extracellular matrix which formed in and around microsphere depots. Low magnification views of UB-PLGA, PLA and B-PLGA microsphere implant sites at day 10 show these voids and the resulting pattern of cellular infiltration (Fig. 4a–f). In general, the initiation and resolution of cellular and fibrotic events observed followed the pattern previously described for the tissue response with cellularity preceding fibrosis and fibrosis becoming more organized, densely-packed and acellular with time (Fig. 1). Differences in the pattern of cellular infiltration and fibrotic deposition at UB-PLGA, PLA and B-PLGA microsphere injection sites seemed to be keyed to variations in the timing and nature of cellular events. UB-PLGA depots were completely penetrated by cells and collagen fibers which outlined individual microspheres (Fig. 4a,b). PLA depots showed a lace-like cellular infiltrate and fibrotic deposition similar to that observed for UB-PLGA, however this morphology was restricted to the margin of the depot (Fig. 4c,d). Little or no cellular infiltration occurred at the center of PLA depots, where the polymer appeared to form a continuous mass (annealing). B-PLGA microsphere depots had sharply defined borders with no cellular infiltration beyond that point (Fig. 4e,f), suggesting an annealing of individual microspheres into one large mass may have occurred. Thus, the distinct cellularity and fibrosis patterns observed for these polymer depots were due to differences in the extent of cellular infiltration between individual microspheres.

This tissue response may be due to the chemical properties of the polymers. B-PLGA polymer is more hydrophobic than UB-PLGA. A microsphere depot formulated with a more hydrophilic polymer, such as UB-PLGA, should retain more of the aqueous-based formulation components between the individual microspheres than a depot composed of a more hydrophobic polymer. Thus, the rate of reabsorption of the excess fluid (the aqueous injection vehicle) from the polymer depot should be greatest for B-PLGA and least for UB-PLGA, and may explain differences in cellular infiltration patterns observed for these two polymer systems. It is also possible that a more rapid loss of water from the B-PLGA depots and from the center of the PLA depots could lead to microsphere association and annealing. Thus, annealing may take place secondarily to the loss of water from the injection depot matrix and may, in part, account for the large, smoothed-walled fibrous capsule observed in the case of B-PLGA microspheres. The Tg values are not different for the three polymers under the physiological conditions of total hydration [20], and therefore annealing differences were probably not due to a difference in this physical characteristic. The depot masses typically generated from PLA microsphere injections showed a decrease in the extent of cellular infiltration from the

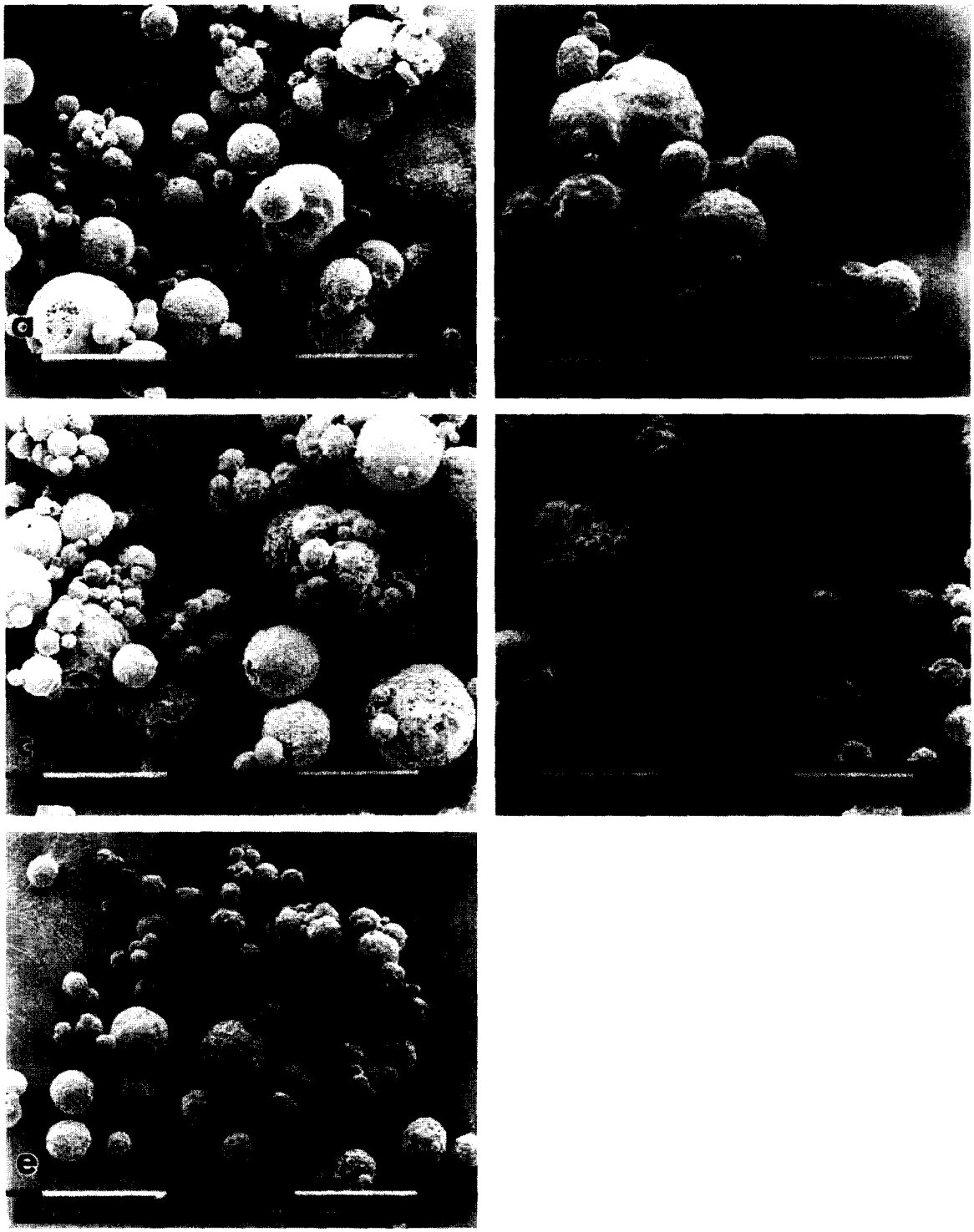

Fig. 3. Scanning electron micrographs of (a) UB-PLGA, (b) PLA, (c) B-PLGA, (d) IGF-I/UB-PLGA and (e) IGF-I/B-PLGA microspheres showing size distribution and surface properties. Bar = 0.1 mm.

outer margin to the center (Fig. 4c,d). Smaller pockets of PLA microspheres, however, showed complete cellular infiltration throughout the margin to center axis of the depot showing a pattern of cellular infiltration similar to UB-PLGA (data not shown). Thus, there may be some critical set of parameters related to polymer hydrophobicity, quantity of microspheres injected and injection vehicle composition such that microsphere formulations could be designed to allow for the desired degree of cellular infiltration.

Cellular and fibrotic events at the surface of PLA depots followed a similar pattern and time course as those observed for UB-PLGA, but the center of PLA depots remained clear of cells and collagen (Fig. 4c,d).

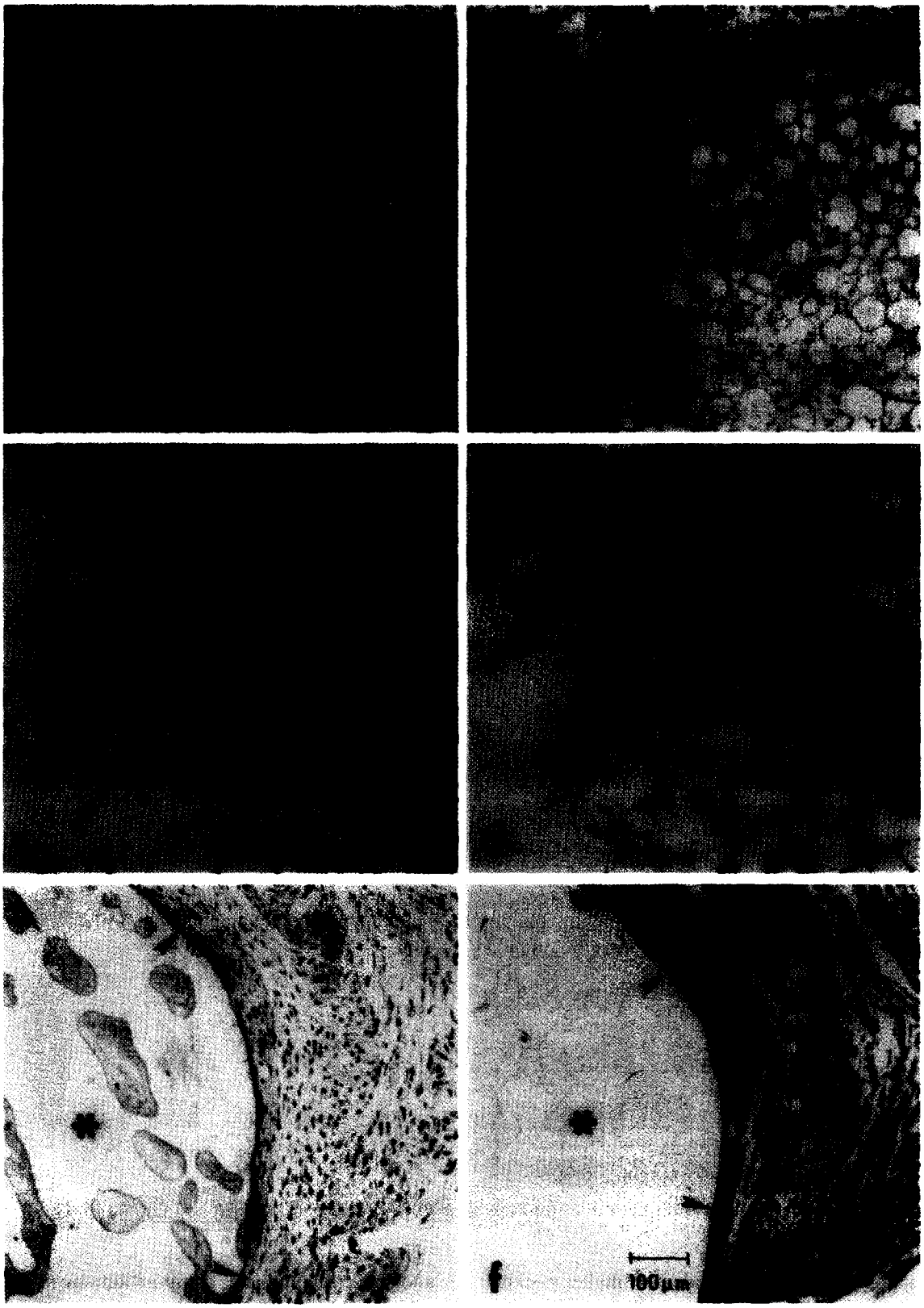

Fig. 4. Depots of UB-PLGA (a,b), PLA (c,d) and B-PLGA (e,f) microspheres at day 10 were prepared and stained with hematoxylin and eosin (HE, left half) or with Masson's trichrome (TC, right half). Note differences in extent and distribution of cells and collagen associated each polymer depot. Voids (asterisks) represent polymer location prior to extraction during sample preparation. In (a), arrow points to FBGC and in (c–f), arrowheads denote the extent of the cellular infiltrate. Residual polymer is observed in (e). All micrographs are the same magnification (100 ×).

Although PMN infiltration was extensive at the periphery of B-PLGA depots at day 3, this intense cellular response did not progress into the depot itself during the 30 day time course. A substantial macrophage population was observed in B-PLGA depots by day 7, but FBGCs were rarely observed at any time. While differences in cellular infiltration patterns (and subsequent fibrosis) at depots appeared to result from variations in polymer surface area accessible to inflammatory cells, differences in cellular infiltration into depots did not seem to correlate with the progression or resolution of inflammation and fibrotic events adjacent to depots. An organized, compact fibrous capsule formed more rapidly around the B-PLGA depot compared to those formed with UB-PLGA and PLA. Thus, the impact of polymer hydrophobicity in our studies was consistent with a previous report where the size and shape of the implanted delivery system modified the inflammatory and healing stages of the tissue response [4].

The hydrolysis of polylactide depots should be controlled by some combination of inherent hydrolysis rate of the polymer and by enzymatic activities of phagocytic cells at the injection site. Further, the hydrophobic nature of the polymer may play an important role in implant fate through several mechanisms: exclusion of water, decreased surface area and diminished cellular penetration. Thus, the relative significance of inherent versus cell-mediated polymer hydrolysis in vivo is difficult to determine. Many investigators have described a disparity between in vitro and in vivo release rates of subcutaneously implanted bioerodible polymeric delivery devices, where the in vivo release is more rapid than that seen in vitro [21,22]. This discrepancy may be due, in part, to the action(s) of phagocytic cells at an implant site which cannot be mimicked in an in vitro setting. The annealing of microspheres may also hinder diffusion of a drug and thus alter the in vivo release profile. The incorporation of a quantifiable agent and measuring its appearance in the circulation, compared to its in vitro release, could allow a rough assessment of how much effect cellular hydrolysis and polymer depot surface area has on the delivery system release.

3.3. Insulin-like growth factor-I loaded UB-PLGA and B-PLGA microspheres

Human IGF-I is a 70 amino acid protein with a molecular mass of 7648 Da. It is 98% homologous with rat IGF-I (only three amino acids are different) and does not produce an immune response when administered continuously to rats [23]. UB-PLGA microspheres contained 15.5% rhIGF-I which released in vitro with roughly zero-order kinetics of 5% per day following an initial burst of 25% and a lag phase of 7 days. B-PLGA microspheres contained 14.8% rhIGF-I which released at approximately 1% per day following an initial burst of 50% and a lag phase of 10 days. The tissue response at depots of UB-PLGA and B-PLGA microspheres loaded with rhIGF-I was indistinguishable from that observed for implants of UB- and B-PLGA microspheres. Unlike previous histological sections where extraction of microspheres during processing left voids where polylactide polymer had resided (Fig. 4), rhIGF-I precipitated as a red particulate at sites where protein-loaded microspheres were located (Figs. 5 and 6).

3.4. Pharmacological agents and assessment of the tissue response

We next sought to determine whether the tissue responses to polylactide microspheres loaded with rhIGF-I could be modulated pharmacologically using a sustained infusion system at the site of depot implant (Fig. 2). Agents co-administered with the rhIGF-I loaded microspheres are shown in Table 1 and doses for these agents were selected from previous in vitro and in vivo data. The approach of using an Alzet® minipump, rather than preparing individual microsphere preparations co-loaded with rhIGF-I and each of these agents, was taken for several reasons. One reason is that the release profiles of each of the agents were likely to be very different and probably different for each agent between the UB- and B-PLGA systems. Another reason is that these agents had previously been delivered using these pumps and were thus chemically compatible with them. Concerns about the chemical compatibility of some of these agents with solvents used to prepare UB- and B-PLGA polymers was thus alleviated.

Early biological events of the tissue response occur as a consequence of the unavoidable tissue damage which results from implantation and the body's efforts to eliminate, or wall off, implanted foreign materials. Therefore, some of the agents listed in Table 1 were chosen on the basis of their putative action during wound healing while others were identified as potential modulators of biological events associated with early stages of the tissue response. Some agents were selected to block while others were selected to enhance a response. Although murine forms of protein agents were used whenever available, some human proteins which could be immunogenic were used. Since previous studies examining the immune potential of a human protein continuously infused into a rat showed neutralizing immune responses after 14–16 days ([24], we limited the duration of our infusions to 2 weeks.

The data presented here on the effect of co-administered agents on the tissue response to the two types of microspheres is qualitative. Cage implant systems have been developed [25] to provide more quantitative infor-

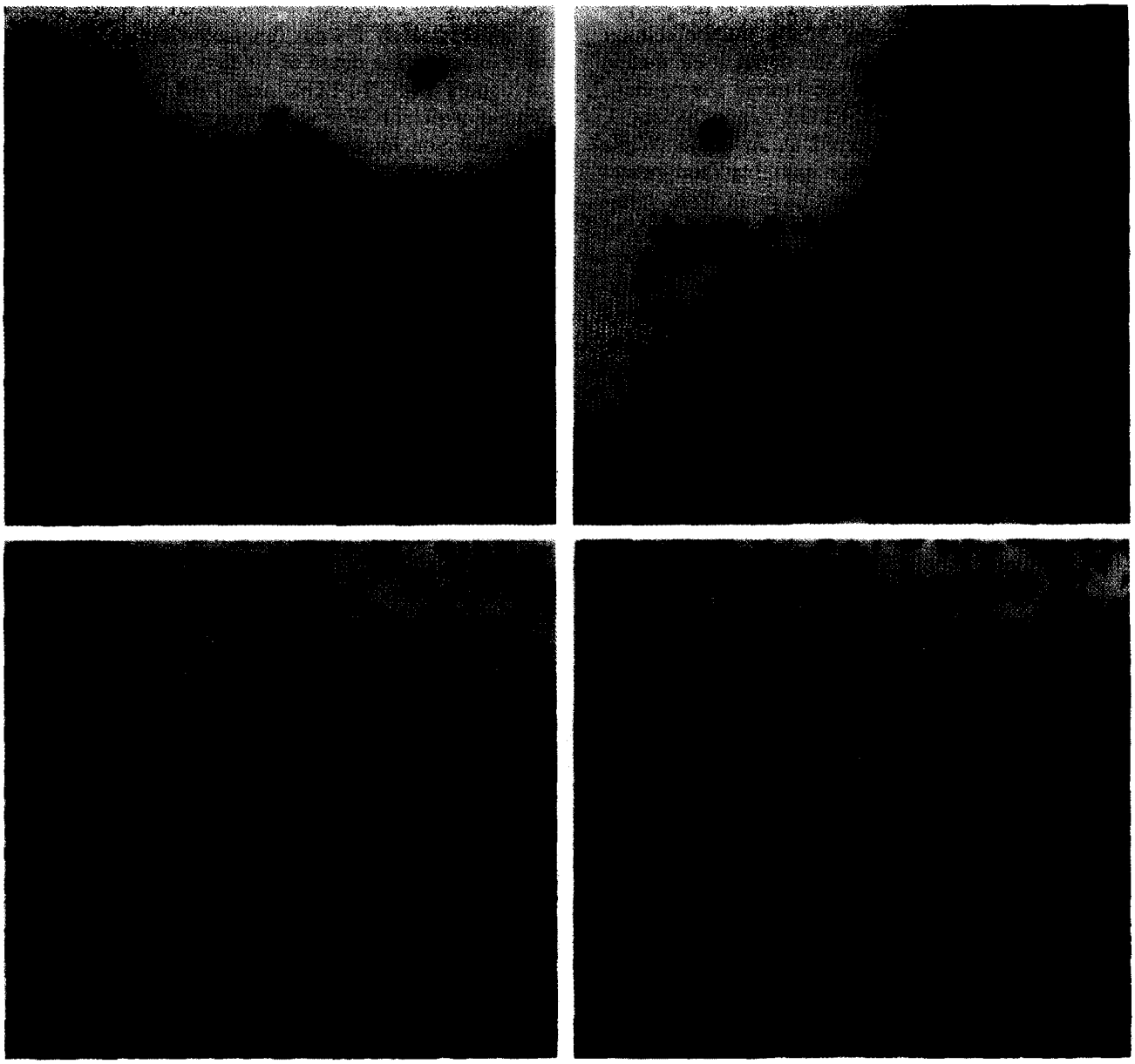

Fig. 5. Representative examples of cellular events associated with polymer depot sites and infused test agents. Sections were stained with hematoxylin and eosin (HE). Voids (asterisks) represent polymer location prior to extraction during sample preparation. In (a) and (b) arrowheads denote location of fibroblasts. Arrows in (c) and (d) point to the numerous macrophages. Stars in these sections indicate regions of FBGCs. See text for explanation of rating system used to describe responses. Magnification, 200 ×.

mation of various biological responses (e.g. enzymatic action and pH changes) but these do not easily lend themselves to the study of microspheres. Our system of analysis was clearly capable of identifying differences in the relative amount of cellularity and the types of cells involved in that response and showed that the two types of polylactide microspheres containing rhIGF-I were biocompatible. We could also assess the extent of fibrosis as well as the density and organization of collagen fibers in and around the injection site.

In order to better evaluate the impact of agents listed in Table 1 on the tissue response, we established a semi-quantitative system to describe the extent of cellular and fibrotic events at polymer depots. Sections stained with hematoxylin and eosin (HE) were scored for cellularity on a relative scale of 0–4, where 0 indicated no response and 4 represented a severe cellular response. Sections stained with Masson's trichrome (TC) were similarly evaluated for fibrosis on a relative scale of 0–4, where 0 indicated no fibrosis and 4 represents extensive fibrosis. Fibrous tissue organization was also evaluated qualitatively and designated as either R for random deposition with no clear organization, L for loose organization that is not densely laid down, O for an organized deposition, or D for a dense, well-defined organization. Some samples displayed both

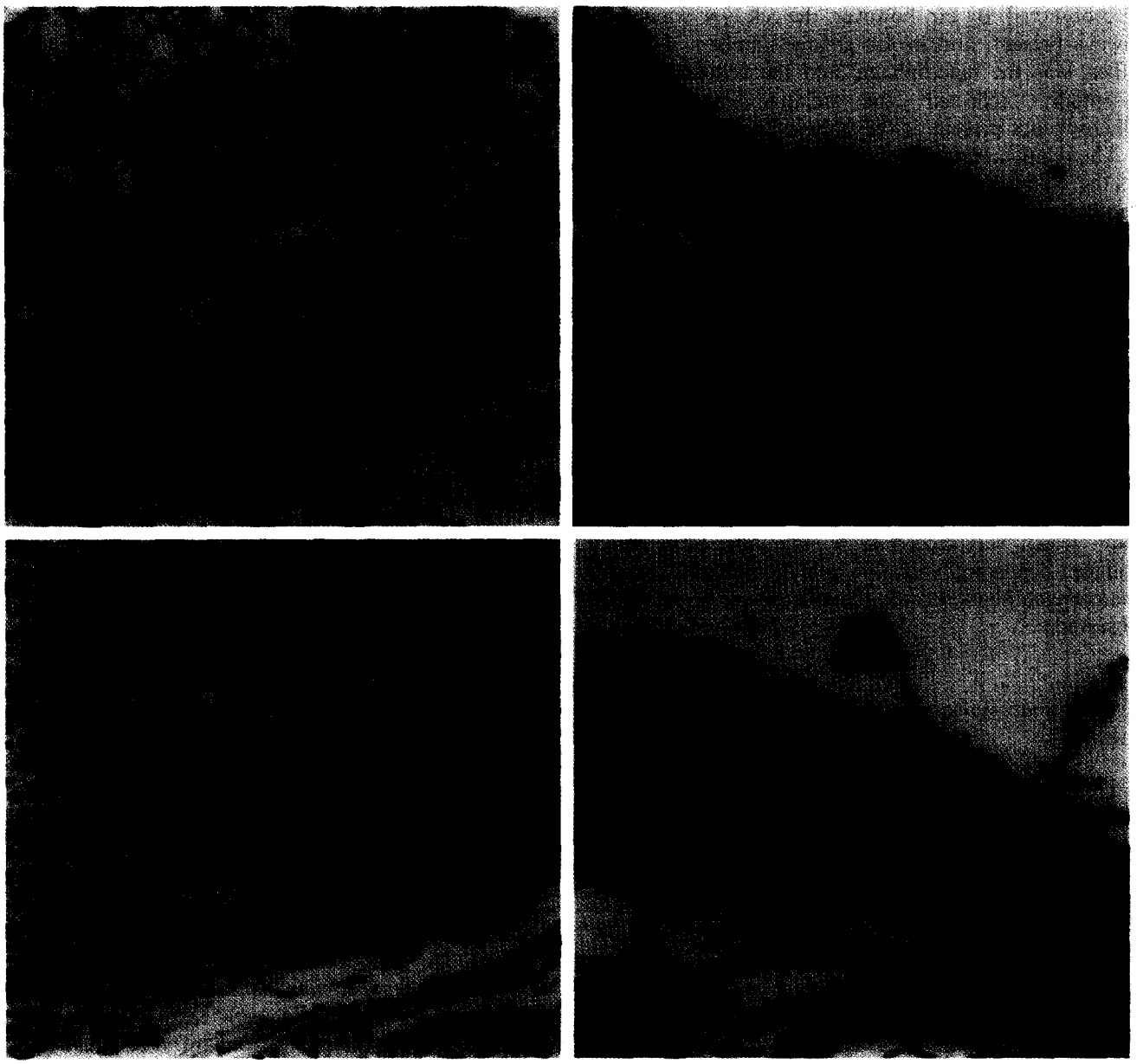

Fig. 6. Representative examples of fibrotic events associated with polymer depot sites and infused test agents. Sections were stained with Masson's trichrome (TC). Voids (small asterisks) represent polymer location prior to extraction during sample preparation. The arrowheads in (c) point to a region of organized collagen fibers. The double-ended arrow in (d) indicates the width of the collagen band. See text for explanation of rating system used to describe responses. Magnification, 200 ×.

random organization with areas of organization, and were designated as RO. Others showed loosely organized areas along with very organized areas, receiving the label of LO. Representative examples depicting each of the levels and categorizations of observed cellular and fibrous responses are shown in Figs. 5 and 6, respectively.

Fig. 5, showing examples of microsphere depots which have been scored from 1–4, illustrates the relative densities of cells not normally present in large numbers in these tissues. Macrophages and fibroblasts are normally seen in tissue sections, but not to the degree seen in the tissue proximal to the microsphere depots. Fig. 5a,b exhibiting low cellularity, rated 1 and 2 respectively, show spindle-shaped fibroblasts with small numbers of macrophages. Fig. 5c,d, representing ratings 3 and 4, respectively, show high cellularity, with large numbers of macrophages and regions of foreign body giant cells, directly at the tissue-microsphere interface. At higher magnification than that shown in this figure other cell types were seen around the depot sites, depending on the time of sampling. Most notably PMNs were observed early in the time sequence. Eosinophils, basophils, mast cells and plasma cells were

also observed. By far, however, the cell type which was always present, and in far greater numbers than any other, was the macrophage, and the cellularity score essentially reflected the relative numbers of macrophages present at the polymer depot.

The relative amounts of fibrosis and the organization of the collagen fibers are shown in the representative sections of Fig. 6. Collagen fiber density which was sparse and not different in structure from the surrounding tissue was rated as a 1 (Fig. 6a). Sparse collagen density typically showed no organization and was scored as random. As the collagen fiber density increased to the rating of 2, it generally became somewhat organized, but loosely, as is seen in Fig. 6b. As the density of collagen deposition became even heavier (to the level of a 3 rating), a defined, organized pattern of fiber orientation typically developed (Fig. 6c). Finally, the pattern of collagen deposition at the tissue-polymer interface was a broad, well-defined, acellular band which was scored as 4 (Fig. 6d). Such heavy collagen deposition with few cells interspersed between the collagen fibers showed the most dense organization observed.

3.5. *Pharmacological manipulation of the tissue response*

Tissue sections of rhIGF-I/UB-PLGA depots (Fig. 7) and rhIGF-I/B-PLGA depots (Fig. 8) were evaluated over a 30 day time course for cellularity and fibrosis as described above. Pharmacological agents were introduced for the initial 14 days of the time course. Without pharmacological intervention, rhIGF-I/B-PLGA depots had fibrosis focused at the periphery while rhIGF-I/UB-PLGA depots had fibrotic tissue not only around the periphery of the microsphere depot, but also between the microspheres. Although studies with agents infused into the injection sites suggested cellular and fibrotic events could be manipulated pharmacologically, the significant role played by the polymer characteristics in determining the tissue response sequelae was still apparent. For example, the intense cellular response at depots of rhIGF-I/UB-PLGA microspheres was refractive to the bioactive agents tested. Although no infused agent was capable of modulating the cellular response to rhIGF-I/UB-PLGA depots, several agents altered the cellular response associated with rhIGF-I/B-PLGA depots. Further, some agents, such as hRlx-2, showed differences in their action(s) on fibrosis when infused into rhIGF-I/B-PLGA versus rhIGF-I/UB-PLGA depots. Although an inhibition of collagen matrix maturation was observed for both, the extent of collagen distribution was limited initially at rhIGF-I/B-PLGA depots but enhanced at depots of rhIGF-I/UB-PLGA.

Only general trends for the actions of infused agents are discussed above. Additionally, some agents produced spikes of response at only one or two time points and did not present clear trends. Some response spikes in either cellularity or fibrosis coincided with a change between day 10 and day 17. Since agents were infused for 14 days, responses to the withdrawal of an agent would occur at this time. For example, rhTGF-β1 can inhibit cell growth [26,27] and the burst in cellularity seen at day 17 in rhIGF-I/B-PLGA depots may reflect the removal of this suppressive action. A similar spike in cellularity in rhIGF-I/B-PLGA and fibrosis in rhIGF-I/UB-PLGA depots at day 17 was observed for rhbFGF. This growth factor has been shown to modulate cell events in wound healing and angiogenesis [5,28] and these spikes might relate to complex events which follow its withdrawal.

It is also possible that these spikes in tissue response events could have resulted from assessment of non-representative sections or samples from a group. It was not possible to insure that the sections of depots used to evaluate each group were equivalent for location and tissue response progression. Alternately, these spikes may also have been part of a trend, but the trend was too rapid to be detected by the limited number of time points possible in our study design. Further, individual animal variation could account for some of these uncertainties. If it were possible, repeated sampling from the same depot site, which did not alter the progression of the tissue response at that site, would have been optimal.

A more consistent result was observed for groups treated with the anti-inflammatory agent dexamethasone (Dex) where a decreased fibrosis in rhIGF-I/UB-PLGA depots was demonstrated suggesting a correlation between fibrosis and inflammatory events. Although rmTNF-α has been shown to have many actions related to the inflammatory response [29,30], this cytokine is also capable of decreasing collagen synthesis [31]. It appears that this latter effect dominated at UB-PLGA depots since fibrosis was decreased in the group treated with rmTNF-α. Leva, which can potentiate the immune response in immuno-compromised animals [32], also diminished fibrosis, while Csp-A, an immuno-suppressive agent [33], did not produce a striking effect on fibrosis. The lack of response to Csp-A might reflect an insufficient dose; however, the effect of Leva treatment is enigmatic, as it is not clear how an agent which could potentiate an immune response might produce a decrease in fibrosis. The observation that rhTGF-β1 enhanced the density of the collagen matrix at rhIGF-I/UB-PLGA depots was consistent with previous studies showing an increase in fibroblast migration [34] and collagen synthesis [35] by this growth factor. Similarly, the inhibition of collagen matrix maturation by hRlx-2 was anticipated since this

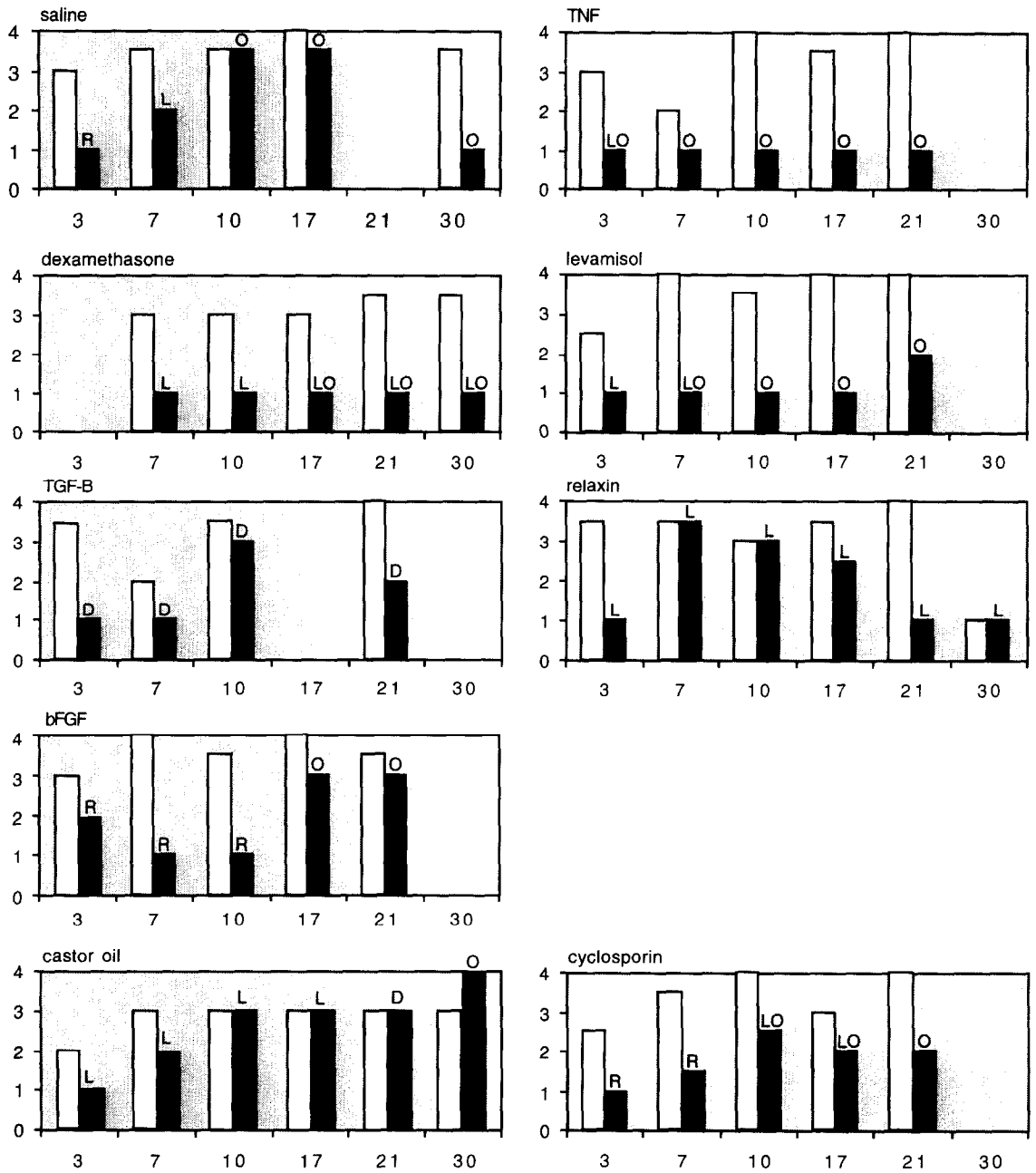

Fig. 7. Time course of cellular and fibrotic responses at depots of rhIGF-I loaded UB-PLGA microspheres. Injection sites were infused with agents (see Table 1) for 14 days. Clear bars represent the degree of cellularity; filled bars represent the relative amount of fibrosis. See text and Fig. 6 for details of the rating system used.

protein has been shown to decrease collagen synthesis and secretion and increase collagenase activity [36–38].

Both cellular and fibrotic events at rhIGF-I/B-PLGA depots could be manipulated by agents described in Table 1. With these microspheres, the immuno-suppressive agent Csp-A reduced fibrosis while the immuno-potentiator Leva had a more subtle action. Dex partially reduced the cellular response in rhIGF-I/B-PLGA depots while rmTNF-α appeared to accelerate the cellular response in rhIGF-I/B-PLGA depots, consistent with their anticipated actions of inhibiting and stimulating inflammation, respectively. Although there is no clear explanation, however, how both rhTGF-β1 and hRlx-2 could impede the cellular response at rhIGF-I/B-PLGA depots, human relaxin inhibits 3T3 fibroblast cell division [39] and TGF-β1 can inhibit fibroblast growth [40]. The actions of these two molecules are typically considered to have opposing responses, as observed for the stimulation of collagen matrix maturation by rhTGF-β1 [26,27] or its inhibition by hRlx-2 [37,38] Similar to hRlx-2, rmTNF-α also appeared to slow collagen matrix maturation, consistent with an action of decreased collagen synthesis [31].

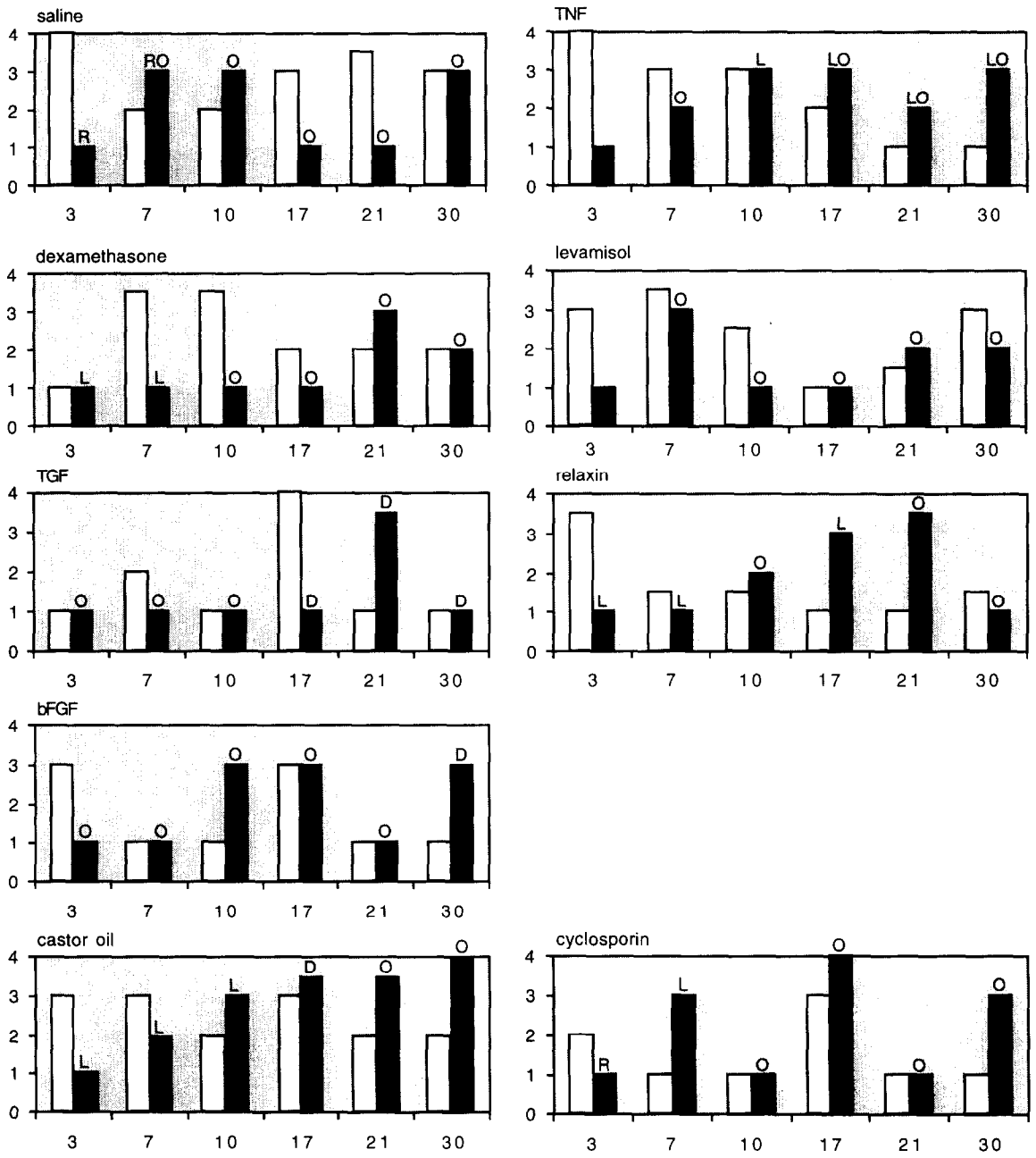

Fig. 8. Time course of cellular and fibrotic responses at depots of rhIGF-I loaded B-PLGA microspheres. Injection sites were infused with agents (see Table 1) for 14 days. Clear bars represent the degree of cellularity; filled bars represent the relative amount of fibrosis. See text and Fig. 6 for details of the rating system used.

Growth factors, as well as cytokines, can have pleiotropic as well as overlapping effects [41], challenging any simple interpretation of results obtained with these agents. For example, rhTGF-β1 can induce a number of actions involving cell growth, cell differentiation, cell adhesion and cell migration [42]. TGF-β1 can have opposing actions on endothelial cell proliferation at different concentrations [43] possibly due to the potential of rhTGF-β1 to activate two different signal transduction pathways [44]. Tissues can also produce a local release of cytokines, lymphokines and growth factors [41] with endogenous rhTGF-β1 being produced in response to tissue damage [45] and possibly by activated macrophages [46]. Factors such as rhTGF-β1 may also not work in the most direct route. For example, bFGF can be released from tissues following trauma and has been shown to enhance myelopoiesis by modulating the inhibition by rhTGF-β1 [47]. Since both bFGF and TGF-β1 modulate angiogenesis [42], these interactions can be further complicated. Finally,

the stimulation of type I and type III collagen by rhTGF-β1 can be depressed by hRlx-2 [37], suggesting complex interactions between these factors as well.

4. Conclusions

A model has been described for the in vivo assessment of the tissue response associated with subcutaneous implants of polylactide microspheres. This model was used to compare responses to microspheres prepared from either UB-PLGA, PLA or B-PLGA. Depots of these polymers were produced by the subcutaneous injection of an aqueous-based formulation which produced a mass of microspheres within the fascia underlying the panniculus carnosus. This depot location was easily accessible for recovery of microspheres and associated tissues as well as for the infusion of bioactive agents intended to modulate the events of the tissue response. Our results demonstrate differences in cellular and fibrous responses which could be correlated with polymer hydrophobicity. Incorporation of a therapeutically relevant protein, rhIGF-I, into UB- or B-PLGA microspheres did not alter the cellular and fibrotic responses to these polymers. Infusion of bioactive agents into the tissue surrounding polymer depots could alter both the pattern and time course of cellular and fibrotic events associated with the tissue response.

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