In vivo biocompatibility studies of poly(D,L-lactide)/poly(ethylene glycol)-poly(L-lactide) microspheres containing all-trans-retinoic acid

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Received 15 October 2001; accepted 23 February 2002

Abstract—Biocompatibility studies of all-trans-retinoic acid (RA)-loaded microspheres were carried out after they were subcutaneously injected into rats. To characterize the inflammatory response to these microspheres, tissue reactions at the implantation site and cell types in the interstices of the microspheres were evaluated for 180 days. On the 15th day, the cross-sectional area of the fibrous capsules surrounding the implantation site of the RA-loaded microspheres was four times larger than that of the control microspheres. The size of the fibrous capsules surrounding the implantation site of the RA-loaded microspheres decreased significantly over a period of 75 days, while the size of the fibrous capsules surrounding the implantation site of the control microspheres remained almost constant throughout the entire course of 180 days. The tissue response to the RA-loaded microspheres was more intensified by the increased extensive cellular infiltration of macrophages, granulation tissue, and fibrosis than that to the control microspheres. The difference in the inflammatory response between the RA-loaded microspheres and the control microspheres was significant for 75 days after implantation. It was suggested that the released RA from the microspheres stimulated inflammatory responses. However, no further enhanced inflammation reactions were detected after RA had been completely released from the microspheres.

Key words: All-trans-retinoic acid; PDLLA microspheres; biocompatibility; foreign body reaction.

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INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is an aggressive epithelial malignancy that is now the sixth most prevalent neoplasm in the world. In spite of much advancement in treatment modality, its long-term survival rate has remained at less than 50% for the last 30 years, due in part to the frequent development of second primary cancers [1, 2]. Therefore, the use of chemotherapeutic agents to prevent the development of second primary cancers has been studied extensively in animal models of oral carcinogenesis and is under investigation in many clinical trials.

One of the most potential chemopreventive agents is all-trans-retinoic acid (RA), since it plays essential roles in the regulation of cell differentiation and in the proliferation of epithelial tissues [3]. These effects of RA on HNSCC have been proved in vitro and in vivo [4–7]. Its clinical application, however, is strictly limited, due to the induced metabolism by specific P450s in the liver (called 'acute retinoid resistance') [8]. Therefore, in spite of continuous oral administration of RA, its concentration rapidly decreases due to RA catabolism by cytochrome P450s induced by RA. In order to overcome this problem, several drug delivery formulations have been investigated: microemulsions [9], liposomes [10], and nanoparticles [11]. Parenteral administration of RA-encapsulated liposomes, in particular, has shown decreased metabolism of RA in the liver.

In our previous studies, RA-loaded biodegradable microspheres were developed in order to prevent 'acute retinoid resistance' [12]. When RA is administered orally, the route of passage is the portal vein, liver, systemic blood stream, and then target organs. So, the absorbed RA could be metabolized in the liver before it reaches the target sites (first-pass metabolism). In contrast, RA released from subcutaneously implanted microspheres would go to the systemic blood stream first and then to the target organs. The rapid induction of specific P450s in the liver could then be reduced. In addition, the controlled release of RA could maintain the concentration of drug in the plasma for a long period. Long-term delivery of RA would therefore greatly improve its chemopreventive effect for a long time, thereby overcoming the acute retinoid resistance in treating second primary cancers. In this study, RA-loaded microspheres were subcutaneously implanted into rats and the tissue reactions to the microspheres at the implantation site were investigated.

MATERIALS AND METHODS

Materials

All-trans-retinoic acid (RA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Poly(D,L-lactide) (PDLLA, Resomer R202) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Poly(vinyl alcohol) (PVA, 98% hydrolyzed, average $M_w$ 13,000–23,000) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Poly(ethylene glycol)-poly(L-lactide) diblock copolymer
(PLE) was synthesized with mono-methoxy PEG (MPEG, $M_n$ 5000) and L-lactide as described in our previous study [12]. The number-average molecular weight ($M_n$) and the molecular weight distribution of the synthesized PLE were 32 500 Daltons and 1.46, respectively. In our previous study, we reported that PLE in the microspheres enhanced drug release from the microspheres and improved the dispersity of the microspheres in phosphate buffered saline (PBS) [12]. PDLLA microspheres are aggregated due to the hydrophobic property of PDLLA and a surfactant would be necessary to disperse PDLLA microspheres in PBS. However, the microspheres containing PLE were dispersed well in PBS without any surfactants, because the hydrophilic PEG blocks of PLE were located at the microsphere surfaces and made the surface hydrophilic.

**Preparation of PDLLA microspheres**

The microspheres were prepared by the solvent evaporation technique in an oil-in-water emulsion. PDLLA (4 g), RA (0 wt% for control microspheres and 10 wt% for RA-loaded microspheres), and PLE (8 wt%) were dissolved in 50 ml of dichloromethane. This mixture was poured into 600 ml of an aqueous solution containing 2% (w/v) of PVA, while mixing vigorously using a mechanical stirrer (IKA LABORTECHNIK, Selangor, Malaysia) at 1000 rpm. After mechanical stirring for 10 min, the resulting suspension was gently stirred for 3 h at 40°C with a magnetic stirrer to evaporate the dichloromethane. The microspheres were collected by centrifugation at 12 000 rpm for 10 min. The obtained microspheres were washed with distilled water, centrifuged four times, and freeze-dried. The morphology of the dried microspheres was observed by a scanning electron microscope (SEM, JSM-5800, JEOL, Tokyo, Japan).

All microspheres were sterilized by the gamma-irradiation method before implantation into rats. Gamma-irradiation sterilization was performed in the open air at room temperature in a $^{60}$Co source with a total dose of 25 kGy. The sterilized microspheres were then dispersed in a sterile, non-pyrogenic normal saline solution at a concentration of 250 mg/ml and were collected in sterilized disposable syringes with 20-gauge needles.

**Evaluation of tissue reactions**

To evaluate tissue reactions to the microspheres, a total of 48 female F344 rats were purchased from Japan SLC Inc. (Hamamatsu City, Japan) and fed a basal diet and tap water *ad libitum*. They were divided into two groups (i.e. the control microsphere group and the RA-loaded microsphere group) when their weights reached 200–300 g. Rats in the RA-loaded microsphere group were subcutaneously injected with RA-loaded microspheres on the back of the neck. A dose of RA-loaded microspheres was determined to be 50 mg RA/kg according to our previous studies. In the chemoprevention study performed for 22 weeks, cancer in the oral cavity was induced by administering water-soluble carcinogen into F344 rats; the
occurrence of invasive squamous cell carcinoma in the tongue and hard palate was significantly prevented by injecting the microspheres at 50 mg RA/kg. From a sub-acute toxicology study, systemic toxic effects were mild at this dose (data not shown). In the control microsphere group, rats were given the same amount of control microspheres as the RA-loaded microsphere group.

Each group of animals was killed with an overdose of ether anesthesia at 15, 30, 45, 60, 75, 90, 120, and 180 days after implantation. In this study, the gross and microscopic analyses of all implantation sites were carried out as described by Yamaguchi and Anderson [13, 14]. The implantation sites were cut with razor blades at the respective time periods mentioned above and fixed in 10% buffered formaldehyde solution. The fixed tissues were embedded in paraffin and sectioned with a microtome. The samples were stained by two methods of histopathological evaluation: hematoxylin and eosin (H&E) for the cellularity and number of cells, and Masson’s Trichrome (Masson) for fibrous capsule formation and collagen deposition.

The size of the fibrous capsule formed was evaluated by measuring its cross-sectional area. The tissue reactions of the implantation site were evaluated by investigating the following factors: liquid exudates inside the fibrous capsule; the types of cells around the microspheres; foreign body giant cell (FBGC) formation; and collagen deposition in the fibrous capsule formed.

RESULTS

Tissue reactions to the control microspheres

Although hydrophobic RA was well dispersed in the hydrophobic microspheres, the loading amount of PLE in PDLLA microspheres was limited, since PLE contains hydrophilic PEG. In our previous study, the optimum conditions for the microsphere preparation were 8 wt% of PLE and 10 wt% of RA [12]. Microspheres containing RA showed a smooth and dense surface structure as shown in Fig. 1 and their sizes ranged from 20 to 100 μm. In our previous study, analyses of the sterilized microspheres by gel permeation chromatography (Waters LC system coupled to a Waters 410 Differential Refractometer, Waters Co., Milford, MA, USA), 1H-NMR spectrometry (JEOL JNM-LA 300 WB, 300 FT-NMR, Tokyo, Japan), and HPLC (Hitachi HPLC system, D-7000 series, Hitachi Ltd., Tokyo, Japan) showed that the molecular weight of the polymer was slightly decreased by 850 Daltons (~5.3% decrease) and the chemical structure of RA appeared intact during gamma irradiation.

On the 15th day, thin fibrous capsules of round or spheroid shape were observed at the interface of the subcutaneous tissue and the implantation site (Figs 2a and 3a-c). The inner part of the fibrous capsule was filled with microspheres and liquid exudates. In the fibrous tissue formed, some parts of the injected microspheres were trapped. The trapped microspheres were surrounded by macrophages, which
formed the major cell component, and fibroblasts and a few FBGCs. Collagen deposition was low. The boundary between the newly formed fibrous tissue layer and the liquid exudates was occupied prominently by fibroblasts, collagen, newly
formed blood vessels, and infiltrating monocytes (Fig. 3c). No polymorphonuclear (PMN) cells were observed in the implantation site.

On the 30th day, the liquid exudates in the inner part disappeared and instead, the inner part was completely filled up with fibrous tissue (Figs 2b and 3d–3f). More FBGCs and macrophages were formed, compared with the results from the 15th day. In the fibrous tissue, minimal collagen deposition was observed. Thereafter, the extent of the inflammatory response increased slightly, but significant interval changes were not observed up to the 60th day (Figs 4a and 4b).

On the 75th day, slightly increased numbers of FBGCs and more collagen deposition were seen compared with the 60th day (data not shown) and were maintained up to the 180th day without any significant increase in the tissue reactions. On the 180th day, a large number of macrophages still remained in the interstices of the microspheres and FBGCs were observed on the microsphere surfaces (Figs 4d–4f). Degradation of the microspheres was observed at this time.
Tissue reactions to the RA-loaded microspheres

On the 15th day, a thin fibrous capsule of round or spheroid shape was observed at the interface of the subcutaneous tissue and the implantation site (Figs 2c, 5a, and 5b). Collagen deposition was low, but it was relatively prominent in the region where the newly formed fibrous tissue contacted the subcutaneous tissue, and also in the region between the fibrous tissue layer and the exudate (Fig. 5b). For RA-loaded microspheres, blood vessels were scarcely seen near the boundary of the fibrous tissue layer and the exudates (Fig. 5c). Interstices of the trapped microspheres in the newly formed fibrous tissue were filled with many macrophages, which formed the major cell component, and fibroblasts and a few FBGCs (Fig. 5d). The inner part of the fibrous capsule was filled with some bloody exudates containing many red blood cells and PMN cells (Fig. 5e).

On the 30th day, more FBGCs and collagen deposition in the fibrous tissue were found compared with the 15th day (Figs 6a–6c). A band consisting of fibroblast and collagen was thickened. As before, blood vessels were scarcely seen near the boundary of the fibrous tissue layer and the exudates at this time (Fig. 6d). Bloody exudates in the inner part still remained and had almost disappeared by the 45th day, as shown in Fig. 6e. A thick collagen band was observed to be growing (Figs 6e and 6f). In the inner part of the fibrous capsule, many monocytes and a thin band of fibroblasts were observed (Fig. 6g).

On the 60th day, the inner part of the injected site was completely filled up by the fibrous tissue (Figs 2d and 7a–7c). Macrophages were the major cell type in the fibrous tissue and the number of FBGCs had increased. Extensive collagen deposition in the interstices of the microspheres was also noted. On the 75th day, it was observed that the formation of large FBGCs (> 20 nuclei) and collagen deposition were greater than on the 60th day (Figs 7d–7f). The size of FBGCs on the surface of the RA-loaded microspheres was larger than that of the placebo microspheres on the 180th day (cf. Fig. 4f with Fig. 7f). There was no significant change in the tissue reactions up to the 180th day. On the 180th day, many large FBGCs, many macrophages, fibroblasts, and extensive collagen deposition could be seen (Figs 8a–8d).

The cross-sectional area of the fibrous capsules of the RA-loaded microspheres at 15 days was about four times larger than that of the control microspheres (Fig. 9). The cross-sectional area of the fibrous capsules had strongly decreased by approximately 1.6 times during the period of 75 days. Thereafter, there was no obvious change in the area from 75 to 180 days.
Figure 3. Histology of the control microsphere subcutaneous implantation sites at 15 and 30 days: (a) 15 days, H&E stain (40×). Note the presence of capillaries (small arrow) in the region between the fibrous tissue and the inner part. (b) 15 days, Masson stain (40×); (c) 15 days, H&E stain (200×). Magnified picture of the region between the fibrous tissue and the inner part. The arrow indicates red blood cells of the newly formed blood vessels in this region. (d) 30 days, H&E stain (40×); (e) 30 days, Masson stain (40×). Note the filling up of the inner part with fibrous tissue (large arrow). (f) 30 days, H&E stain (200×). (This figure is published in colour on http://www.vsppub.com/jconts/JBS)
Figure 3. (Continued).
Figure 4. Histology of the control microsphere subcutaneous implantation sites at 60, 120, and 180 days: (a) 60 days, H&E stain (40×); (b) 60 days, Masson stain (40×); (c) 120 days, H&E stain (200×); (d) 180 days, H&E stain (40×); (e) 180 days, Masson stain (40×); (f) 180 days, H&E stain (200×). Arrows indicate degrading microspheres and the infiltration of cytoplasm of macrophages or FBGCs into the degraded part of microspheres. (This figure is published in colour on http://www.vsppub.com/jconts/3BS)
Figure 4. (Continued).
Figure 5. Histology of the RA-loaded microsphere subcutaneous implantation sites at 15 days: (a) H&E stain (40×); (b) Masson stain (40×). (c) Magnified picture of the region between the fibrous tissue layer and the bloody exudates, H&E stain (200×). Note that blood vessels were scarcely seen near this region. (d) Microspheres trapped in the fibrous tissues, H&E stain (200×). (e) The inner part of the fibrous capsule, H&E stain (200×). (This figure is published in colour on http://www.vsppub.com/jconts/JBS)
Tissue reactions to RA-loaded microspheres

DISCUSSION

The extent and the duration of inflammatory reactions to an implanted material can be affected by several factors such as the size and degradation of the implanted material, and the biological effects of the released drug. The size of the microspheres in this study was larger than 20 μm. Thus, there was no sign of phagocytosis by macrophages, as Tabata et al. [15] reported. They showed that microspheres larger than 10 μm were precluded from phagocytosis by macrophages [15]. FBGCs were formed within 15 days by continuous irritation of the microspheres. They were seen during the 180 days of the experiment because the size of the microspheres did not decrease sufficiently in this period [13, 14, 16]. The different degradation rates of the matrix polymer might be a factor causing different inflammatory responses in some cases. However, the degradation rate of the PDLLA microspheres did not seem to be a main factor affecting the inflammatory response. For copolymers of glycolide and d,l-lactide, it was reported that the degradation of PDLGA and their
Figure 6. Histology of the RA-loaded microsphere subcutaneous implantation sites at 30 and 45 days: (a) 30 days, H&E stain (40×); (b) 30 days, Masson stain (40×). The layer of fibroblast and collagen at the boundary was thickened compared with 15 days and collagen deposition was severe at the boundary layer. (c) Microspheres trapped in the fibrous tissue at 30 days implantation site, H&E stain (200×). (d) Magnified picture of the region between the fibrous tissue layer and the bloody exudates at 30 days, H&E stain (200×); (e) 45 days, H&E stain (40×). Note that the bloody exudate had almost disappeared. (f) 45 days, Masson stain (40×). (g) The inner part of the fibrous capsule at 45 days, H&E stain (200×). Many monocytes and a thin band of fibroblast can be seen. (This figure is published in colour on http://www.vsppub.com/jcorts/JBS)
Figure 6. (Continued).
Figure 6. (Continued).

Figure 7. Histology of the RA-loaded microsphere subcutaneous implantation sites at 60 and 75 days:
(a) 60 days, H&E stain (40×). The inner part of the fibrous capsule was completely filled up.
(b) 60 days, H&E stain (200×); (c) 60 days, Masson stain (200×); (d) 75 days, H&E stain (40×);
(e) 75 days, Masson stain (40×); (f) 75 days, H&E stain (200×). Note the large FBGCs on the
microsphere surface. (This figure is published in colour on http://www.vsppub.com/jcontents/JBS)
Figure 7. (Continued).
degradation products did not affect the inflammatory response [13]. RA release from the PDLLA microspheres, therefore, would be a major factor in the extent of the inflammatory response.

In this study, an increased inflammatory response over the course of 180 days was observed in rats that were given RA-loaded microspheres, but the increase was only slight in control microspheres. The increased inflammatory response by RA-loaded microspheres seemed to be related to the cytokine production of activated macrophages. Cytokines such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), produced from activated macrophages, are important regulators of inflammatory reactions. It has been reported that the production of such cytokines is dependent on the concentration of retinoic acid. When the concentration of
Figure 8. (Continued).
RA was in the range of $10^{-8} - 10^{-6}$ M, the production of IL-1 from activated macrophages was stimulated by retinoic acid, whereas the production of TNF-α was inhibited [17–19]. IL-1 has been demonstrated to induce the up-regulation of intercellular adhesion molecule-1 and E-selection, which can mediate leukocyte infiltration into the implantation site [20, 21]. Therefore, the formation of relatively large fibrous capsules at 15 days for RA-loaded microspheres could be due to the intense reflux of immune cells such as neutrophils and macrophages.

On the 15th day, the cross-sectional area of the newly formed fibrous capsules for RA-loaded microspheres was about four times larger than that of the fibrous capsules for the control microspheres. The cross-sectional area of the newly formed fibrous capsules had greatly decreased during the period of 75 days, while the area of the fibrous capsules at the control microspheres remained almost constant throughout the entire 180 days. In addition, the tissue reactions to RA microspheres increased during the period of 75 days, after which time there were no marked changes in the tissue reactions up to 180 days. This may have been because RA was completely released from the microspheres for 60 days. The plasma concentration of RA was maintained for 8 weeks, ranging from $1.4 \times 10^{-8}$ to $7 \times 10^{-8}$ M (data not shown). The physiological level of RA in humans is reported to be $0.65 \pm 0.18 \times 10^{-8}$ M [22].

On the 15th day, a large number of newly formed blood vessels were observed for the control microspheres in the region between the fibrous tissue and the inner exudate part. In contrast, blood vessels at the boundary of the RA-loaded microspheres were scarcely seen. For the RA-loaded microspheres, this could be the reason why there is a lack of blood vessels at the boundary where RA inhibits angiogenesis (i.e. RA inhibits the formation of new blood vessels) in vivo [23]. This inhibitory effect of RA on angiogenesis might delay the healing process at the inner part of the implantation site, and therefore delay the filling up of the inner part of the implanted site.
Since RA is also well known to stimulate collagen synthesis, it would affect the extent of fibrosis at the implanted sites of the microspheres [24, 25]. The extensively deposited collagen at the implantation sites might act as a permeability barrier and restrict the diffusion of drugs [26]. Moreover, a thick band of fibroblasts, which are known to actively metabolize RA [27], was formed at the boundary of the fibrous tissue layer and the exudates from the 30th to the 45th day. The formation of this band and extensive collagen deposition could be major limitations in the use of RA-loaded microspheres. Therefore, combined chemotherapy with a corticosteroid would improve the efficacy of RA-loaded microspheres by inhibiting edema, fibroblast proliferation, and collagen deposition at the implantation sites [28].

Since RA was completely released from the microspheres within 60 days after implantation, the biocompatibility of these implants after 180 days is only related to PDLLA microspheres. In this study, inflammatory responses after 180 days were not investigated since biodegradation and tissue reactions for the PDLLA microsphere over 480 days have already been studied by Visscher et al. [16].

Degradation of the microspheres was also delayed after the complete release of RA, although the PDLLA microspheres were highly biocompatible. In a further study, microspheres having an ideal degradation period within 120 days will be developed for the repeated injections of RA-loaded microspheres.

CONCLUSION

While RA-loaded microspheres caused inflammatory reactions, the surrounding tissues were histologically intact for the entire 180-day experimental period. Irreversible changes, such as necrosis and degeneration of the tissues at the implanted site, were not observed. After RA was completely released from the microspheres within 60 days, no further enhanced inflammation reactions were induced. Therefore, this microsphere formulation of RA can be considered biocompatible when a dose of 50 mg RA/kg is used over a period of 180 days.

Acknowledgements

This study was supported by grants (HMP-98-G-2-050-B) from the '98 Highly Advanced National Projects on the Development of Biomedical Engineering and Technology, Ministry of Health and Welfare, R.O.K. It was also partially supported by a grant from the Engineering Research Center (CAFPoly), the Korea Research Foundation, and the Brain Korea 21 program.

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