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Tissue compatibility of poly(hydroxypropyl glutamate)-prazosin conjugates

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Biocompatibility of an injectable biodegradable drug delivery system for prazosin was investigated in Sprague-Dawley rats by histological studies after subcutaneous injection of poly(hydroxypropyl glutamate)-prazosin (PHPG-prazosin) conjugate particles. The studies showed that (1) the acute inflammatory response to this injectable biodegradable polymeric prodrug system was mild and of only short duration, (2) the chronic inflammation was minimal to zero, (3) the fibrous capsule could be seen starting from 7 days and became more prominent at longer time periods, (4) a collagen network was formed into the injection site after 21 days, (5) the macrophages and foreign giant cells reacted to the globules of conjugate particles, and (6) no adverse reactions were identified. Focal inflammation and the formation of the fibrous capsule around the injection site were the significant histological findings in the histopathological studies. Therefore, it is concluded that the biodegradable injectable PHPG-prazosin carbamate polymeric prodrug system is tissue biocompatible.

Keywords: Biocompatibility; Polymeric prodrug; Prazosin; Biodegradable; Poly(α -amino acid)

Introduction

Controlled release delivery systems can be administered into the human body by oral, transdermal, or parenteral routes. The limitation of the oral route is the relatively short duration of the controlled release device in the body. In contrast, transdermal delivery and implantable or injectable delivery systems can offer long term, continuous delivery of drugs. However, when the

controlled release devices are introduced by parenteral routes, the body and the tissue will respond to the introduction of the device and cause local or/and systemic reaction.

Biocompatibility is defined as "the ability of a material to perform with an appropriate host response in a specific application" [1]. To evaluate the biocompatibility of an implant, the interaction between the tissue and implant as well as the systemic reaction to the implant should be investigated. In general, the studies can be conducted by *in vitro* and *in vivo* experiments. *In vitro* tests usually are performed by using cell culture techniques and *in vivo* studies are carried out by implantation. The latter usually provide a more realistic appraisal of the device.

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As indicated by the definition of biocompatibility, when a controlled release device is present in the tissue of the body, the interaction of the device and the surrounding tissue or the response due to the device must be acceptable or appropriate. For a subcutaneous implantable or injectable system, the evaluation of tissue response to the controlled release device is an important criterion of the biocompatibility. The histological study of the implant or injection site can provide information about the local tissue environment by the observation of the interaction between tissue and the device as well as the resulting tissue reaction which surrounds the device. This environment can affect the drug release from the device and the degradation or absorption of the device.

Studies have been conducted to investigate the biocompatibility of the polymers and their injectable or implantable controlled release delivery systems. The investigation of the histological change as a function of implant time is the most commonly used method in biocompatibility studies of drug delivery systems. Olanoff and Anderson [2] studied the biocompatibility of the subcutaneous implant of tetracycline-hydroxyethyl methacrylate/methyl methacrylate copolymer drug delivery system. Since the device was a trilaminar disk with a hydroxyethyl methacrylate-methyl methacrylate copolymer coating, the tissue response to the device was similar to that of methyl methacrylate polymer and showed no differences between the loaded device and the drug free device. The tissue reactions of poly(DL-lactide) and poly(DL-lactide-co-glycolide) microcapsules were investigated [3,4] and it was concluded that non-adverse-tissue reaction occurred in response to the intramuscular injection of the microcapsules. Yamaguchi and Anderson [5] studied the biocompatibility of a naltrexone delivery system made of poly[L-(+)-lactide-co-glycolide] microspheres. The only tissue change observed in the studies was the focal inflammation around the microsphere and naltrexone was considered to be the causative agent. Anderson et al. [6,7] used a cage implant system to study the dynamic cellular response and enzymatic

change at the subcutaneous implant site for poly(hydroxyethyl glutamine) hydrogel. The inflammatory response to the poly(hydroxyethyl glutamine) hydrogel was observed by analyzing the exudate. Tissue reaction of copolymers of L-leucine, L-aspartic acid, and L-aspartic acid ester after implantation in the subcutaneous tissue were investigated and it was found that the tissue reaction for the hydrophobic polymer resembled that of silicone rubber, whereas the relatively hydrophilic polymer showed more cellular reaction [8].

Ideally, the result of inflammatory response to the controlled release system would be the complete recovery of the tissue. Unfortunately, this is rarely the case for the implantable or injectable systems. However, the application of the biodegradable polymer offers this possibility [4]. Another aspect which should be considered in the case of controlled release systems is that the bioactive agent should release from the device over the desired period of time and at a desired rate.

A long-term drug delivery system for prazosin has been prepared by covalently bound prazosin onto poly(hydroxypropyl glutamate) (PHPG) via a carbamate linkage [9]. Zero order release of prazosin was achieved for three weeks in *in vitro* release studies and *in vivo* studies were performed with New Zealand White rabbits. PHPG-prazosin carbamate conjugate particles were injected subcutaneously into both flanks of the rabbits. Following an initial burst, a nearly constant plasma prazosin concentration profile above 2 ng/ml was maintained for 10 days [9].

In this study, the histological evaluation was conducted in rats after subcutaneous injection of the biodegradable polymeric prodrug, PHPG-prazosin carbamate.

Materials and methods

Animals

The tissue reaction studies were performed on male Sprague-Dawley rats. Twenty-four rats (~200 g) were divided into eight groups and

each group was housed in a cage. The rats were kept at the University of Utah Medical Center Vivarium during the studies and maintained on food and water ad libitum. Light was controlled with 12 h each of day and night.

PHPG-prazosin carbamate conjugate

PHPG reacted with *p*-nitrophenyl chloroformate to form an active intermediate. Prazosin, then, was coupled onto the active intermediate to form a conjugate with carbamate linkage as a labile bond between drug and polymer [9]. The PHPG-prazosin conjugate particles ($20.8 \pm 5.6 \mu\text{m}$) were disinfected with 70% ethanol solution and dried under vacuum. Ten mg of conjugate particles were suspended in a 1 ml syringe with 0.5 ml of saline before injection.

Injection procedure

Twenty-four rats were divided into eight groups. The injection sites on the rats were shaded and wiped with alcohol prep pads (70% isopropyl alcohol). Each rat had 10 mg of PHPG-prazosin conjugate particles (in 0.5 ml of saline) injected subcutaneously into the left dorsal side with an 18G1 needle. The control (10 mg of PHPG dissolved in 0.5 ml of saline) was injected subcutaneously into the right dorsal side of the same rat. The injection sites were marked by a blue marker pen and remarked every week.

Tissue sample collection

Three rats were killed at 1, 2, 4, 7, 14, 21, 28, and 60 days after injection. The injection sites with adjacent tissue were separated by a scalpel with a size of about $3 \times 3 \text{ cm}$. The tissue samples were tacked by pins at the four corners on paraffin blocks to avoid curling and fixed in 10% neutral buffered formalin solution (Sigma Diagnostics, St. Louis, MO) for 3 days. The fixative solution was replaced with fresh 10% neutral buffered formalin solution.

Histological analysis

The injection sites with adjacent tissue were sectioned with a razor blade into 2 to 3 mm in thickness. The tissue sections which contained the injection sites were identified and dehydrated with an increasing gradient of ethanol concentration, infiltrated with xylene, embedded in paraffin, and then sectioned. The slides were stained with hematoxylin and eosin so that the cellularity could be identified and the number of cells counted. Masson's trichrome and Picrosirius Red were used to distinguish the fibrous capsule formation and collagen deposition. No injection sites of the control sample were identified within the respective tissues. The histological evaluation was performed with light microscopy. Slides stained with Picrosirius Red stains were observed under polarized light. Following evaluation of all samples, the most representative injection sites of the conjugate particles for a given time and different stains were used to prepare 35 mm slides and photomicrographs. The slides were taken at both low ($6.3 \times$) and high ($63 \times$) magnification to show the general morphology of the injection site within the tissue and the cellular components during the inflammatory as well as healing responses to the conjugate particles.

Results and Discussion

In general, the tissue reaction of the injectable controlled release devices can be described from the point of view of wound healing. When the controlled release device is implanted or injected, the local tissue is injured [10]. Initiated by the injury, inflammation follows. Consequently, in terms of the appearance of the morphological changes around the devices, the inflammatory reaction is usually seen in tissue response to the controlled release devices [11]. The following events would occur after an implantation or injection of the controlled release system into the body. The acute inflammation is the first response in a series of reactions and per-

Fig. 1. Subcutaneous injection sites of P:HPG-prazosin carbamate particles in rats (1 day after injection). (A) Hematoxylin and eosin stain (6.3 $\times$); (B) hematoxylin and eosin stain (63 $\times$).

Fig. 2. Subcutaneous injection sites of PHPG-prazosin carbamate particles in rats (2 days after injection). (A) Hematoxylin and eosin stain (6.3 $\times$); (B) hematoxylin and eosin stain (63 $\times$).

Fig. 3. Subcutaneous injection sites of PHPG-prazosin carbamate particles in rats (4 days after injection). (A) Hematoxylin and eosin stain ($6.3\times$); (B) hematoxylin and eosin stain ($63\times$).

Fig. 4. Subcutaneous injection sites of PHPG-prazosin carbamate particles in rats (7 days after injection). (A) Hematoxylin and eosin stain ($63\times$); (B) Masson's trichrome stain ($6.3\times$).

sistent inflammatory reaction leads to chronic inflammation [12].

In this study, the histological evaluation of the injection site for a period of up to 60 days was performed to study the biocompatibility by observation of the inflammatory and healing response to PHPG-prazosin conjugate particles. Hematoxylin and eosin stain was used to distinguish the cellular components during acute inflammation, chronic inflammation, granulation, and fibrous encapsulation. The nuclei appeared as dark purple or black, whereas the cellular cytoplasm and proteins were pinkish-red. Collagen components in samples treated with Masson's trichrome stain appeared blue and greenish color (type III collagen) or reddish-orange color (type I collagen) when stained with Picrosirius Red and observed under the polarized light. The results of the tissue response after injection of the conjugate particles are presented and discussed chronologically.

One day: The injection site was located in the subdermal adipose tissue. The particles of conjugate appeared collectively in ovoid shape (Fig. 1A). Acute inflammation, characterized by polymorphonuclear leukocytes, was noted within the injection site and the surrounding adipose tissue (Fig. 1B). Focal areas of exudate were present within the injection site. Capillaries were seen in the adjacent adipose tissue. The overall inflammatory cell response to the conjugate particles was considered as mild.

Two days: Acute inflammation (polymorphonuclear leukocytes) appeared to increase within the injection site, while the exudate decreased in comparison with that of 1 day (Fig. 2). Capillaries and polymorphonuclear leukocytes were also found in adipose tissue surrounding the injection site.

Four days: The injection site was well circumscribed in an ovoid shape and the conjugate particles were contiguous. Polymorphonuclear leukocytes and monocytes were present within the injection site and in the adjacent adipose tissue, but the number of the polymorphonuclear leukocytes decreased in comparison with that of two days. Focal areas of exudate were rare within the

injection site (Fig. 3). Capillaries were identified in the adjacent adipose tissue.

Seven days: The injection site remained contiguous and ovoid in shape. Inflammatory cells and exudate were seen in the injection site. The foreign body giant cells reacted to the small globules of the conjugate particles at the margin of the collective particles (Fig. 4A). The acute and chronic inflammatory response in the adjacent adipose tissue decreased when compared to the previous observations. Capillaries were identified in the adjacent adipose tissue. A thin fibrous capsule developed as shown in the sample stained with Masson's trichrome (Fig. 4B).

Fourteen days: A developing fibrous capsule surrounded and confined the ovoid shape contiguous conjugate particles in the subcutaneous tissue (Fig. 5A). The macrophages and foreign giant cells were found to react to discrete globules of the conjugate particles within the injection site (Fig. 5B). Both the acute and the chronic inflammation were minimal to zero as indicated by the number of polymorphonuclear leukocytes and monocytes.

Twenty-one days: The ovoid shape injection site was well encapsulated by the fibrous capsule (Fig. 6A,B). As seen in 14 days, within the injection site, the macrophages and foreign giant cells were found to react to discrete globules of the conjugate particles, which was well developed and easy to identify (Fig. 6C). In adjacent tissue, capillaries and minimal to zero inflammation were seen. The sample stained with Picrosirius Red showed that fibroblasts had entered the injection site and produced collagen (reddish-orange network in Fig. 6D) within the injection site.

Twenty-eight days: A thicker and denser fibrous capsule circumscribed the injection site (Fig. 7A). The macrophages and foreign giant cells reacted to discrete globules of the conjugate particles within the whole injection site (Fig. 7B). The network formed by collagen in the injection site can be seen in the sample treated with Picrosirius Red stain (Fig. 7C,D). No or minimal inflammation was noted in the adjacent tissue.

Fig. 5. Subcutaneous injection sites of PHPG-pirarazin carbamate particles in rats (14 days after injection). (A) Masson's trichrome stain ($6.3\times$); (B) hematoxylin and eosin stain ($63\times$).

Fig. 6. Subcutaneous injection sites of PHPG-prazosin carbamate particles in rats (21 days after injection). (A) Masson's trichrome stain ($6.3\times$); (B) Masson's trichrome stain ($16\times$); (C) hematoxylin and eosin stain ($63\times$); (D) Picrosirius Red stain ($16\times$).

Fig. 7. Subcutaneous injection sites of PHPG-prazosin carbamate particles in rats (28 days after injection). (A) Masson's trichrome stain (6.3 $\times$); (B) hematoxylin and eosin stain (63 $\times$); (C) Picrosirius Red stain (6.3 $\times$); (D) Picrosirius Red stain (63 $\times$).

Fig. 8. Subcutaneous injection sites of PHPG-prazosin carbamate particles in rats (60 days after injection). (A) Hematoxylin and eosin stain ($63\times$); (B) Picrosirius Red stain ($63\times$).

Sixty days: The injection sites still can be seen in subcutaneous tissue in an ovoid shape. No acute or chronic inflammation was identified either in the injection site or in the adjacent tissue. Macrophages and foreign body giant cells in the injection site were seen reacting to discrete globules of the conjugate particles (Fig. 8A). The fibrous capsule was found surrounding the injection site and small arterioles as well as capillaries were seen adjacent to it. Masson's trichrome and Picrosirius Red stains showed that fibroblasts had entered the injection site and a fine network of collagen was formed within the injection site (Fig. 8B).

Focal areas of acute inflammation were identified for the samples at one day, two days, and four days. Only one of three injection sites at each time period showed this microscopic focal acute inflammation. The other two did not show the injection site inflammatory response. The histological finding was characterized by the presence of polymorphonuclear leukocytes and focal areas of exudate. The focal inflammatory response was focal and minimal.

In general, histologically, the acute inflammatory response to this injectable biodegradable polymeric prodrug system was mild and of only short duration (up to 7 days) and the chronic inflammation was minimal to zero. The fibrous capsule could be seen starting from 7 days and became more prominent at longer time periods, 14, 21, and 28 days. A collagen network was developed into the injection site after 21 days. By 28 days, the inflammatory and healing responses to the conjugate were well-developed with macrophages and foreign giant cells inside the collective conjugate particles. No adverse reactions, such as necrosis, degeneration, and infection, were identified in all samples.

The acute inflammatory response to the conjugate may account for the initial burst of the prazosin in *in vivo* studies shown in the previous publication [9], since the inflammation would decrease the local tissue pH [13,14]. On the other hand, the formation of fibrous capsule can be a barrier that makes the plasma levels of pra-

zosin lower than that predicted from an ideal model. As studied by Anderson et al. [15], the drug release rate was influenced by the development of the fibrous capsule. Determination of the drug concentration at different distances from the release device surface revealed the spatial difference of the concentration of the drug and verified the presence of a diffusion barrier.

Extensive tests have been performed to evaluate the biocompatibility of the nortriptyline polymeric prodrug base on PHPG, PHEG, and copolymer of HPG and valine [16,17]. The studies involved immunological and histological tests. The immunological studies involved immunization of rabbits and guinea pigs followed by skin test, implantation test, agglutination test, precipitation test, agar diffusion test, complement fixation test as well as passive cutaneous anaphylaxis test. Histological evaluation included the observation of the tissue response and the examination of the organs. All immunological tests were negative and tissue reactions were acceptable. Although all tests showed negative results, to draw a conclusion about nonimmunogenicity of the polymeric prodrug and polymer backbone still needs more rigorous and sensitive tests, for example, using radiotracer technique. Dickinson et al. [8] studied the biodegradation of the poly(α -amino acid) hydrogel by subcutaneous implantation and found tissue responses to the poly(α -amino acid) hydrogel were acute and chronic inflammation.

The results of the histological studies in this investigation are similar to the inflammatory and healing response to those seen with naltrexone delivery system made of poly[L(+)-lactide-co-glycolide] microcapsules or microbeads [5]. This conjugate particle system has a tissue reaction commonly seen for nondegradable or very slowly degradable microspheres or microcapsules. It was found that the inflammatory cell density was higher for the naltrexone loaded beads than for the control, poly[L(+)-lactide-co-glycolide] microcapsules or microbeads and was considered to be due to released naltrexone. However, it was not seen in this study.

Conclusions

In the present study the tissue response to PHPG-prazosin conjugate particle was investigated in Sprague-Dawley rats. By studying the histological changes surrounding the injection site, the tissue compatibility of PHPG-prazosin conjugate was evaluated. All injection sites showed mild and short duration acute inflammatory response, and minimal to no chronic inflammation was identified. The duration of the acute inflammatory response and the time of formation of the fibrous capsule can explain the burst effect and the lower plasma concentration in *in vivo* studies [9]. The significant histological findings in this study were the local inflammation and the formation of the fibrous capsule around the injection site. Therefore, it is concluded that the biodegradable injectable PHPG-prazosin carbamate polymeric prodrug system is tissue biocompatible.

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