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Characterization of subcutaneous Contramid® implantation: host response and delivery of a potent analog of the growth hormone-releasing factor

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Abstract

Cross-linked high amylose starch (Contramid®) was investigated as a solid implant for evaluation of host response in mice and as a possible delivery system for a human growth hormone-releasing factor analog (Hex-hGRF) release profile in pigs. Seventy mice were administered subcutaneously one 3 mm diameter Contramid® pellet and host reaction was evaluated over 6 months. Thirty pigs were divided into four groups. All animals of the three implanted groups were administered subcutaneously 15 mg Hex-hGRF, (1) one pure Hex-hGRF implant; (2) four 30/70 w/w Hex-hGRF/Contramid® implants; or (3) eight 15/85 w/w Hex-hGRF/Contramid® implants. The fourth group ($n = 6$) was injected subcutaneously twice daily with 10 μg/kg of Hex-hGRF over 5 consecutive days. Serum insulin-like growth factor-I (IGF-I) was monitored over 1 month. In mice, no adverse reaction occurred after implantation. Macroscopic and microscopic inflammatory reactions were always localized. Polymorphonuclear cells (PMNs) and macrophages predominated within and around the pellets, respectively. Thin fibrovascular septas eventually subdivided Contramid® pellets which were progressively phagocytosed by macrophages. In pigs, serum IGF-I concentrations were increased over a 10 day period in all implanted groups. The initial IGF-I peak observed in the daily injected group was avoided in both Contramid® implant groups but not in the pure Hex-hGRF implant group. These encouraging results warrant the development of Contramid® implants as a sustained delivery system for peptidic drugs.

Keywords: Cross-linked starch; Implant; Biocompatibility; Controlled release; GRF

1. Introduction

Cross-linked high amylose starch (Contramid®) is a water insoluble polymer and a hydrophilic swelling matrice that was originally developed as a controlled release solid oral dosage forms char-

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acterized by a quasi zero order drug delivery over 12–24 h (Lenaerts et al., 1991, 1998). Previous work using degradable starch microspheres administered nasally as dry powder showed an acceptable biocompatibility (Björk et al., 1991). Bioavailabilities of nasal insulin was improved when administered with starch in rats (Björk and Edman, 1988, 1990) and rabbits (Callens and Remon, 2000) as well as nasal gentamicin in sheep (Illum et al., 1988). Degradable starch microspheres with a chemotherapeutic agent have also been used in humans by hepatic arterial injection in order to create embolization and selectively to increase drug exposure to liver tumors (Dakhil et al., 1982). Starch microparticles were less stimulatory to the macrophages in vitro than other polysaccharides (Artursson et al., 1987a). Microparticulate starch has also been shown to be rapidly cleared from blood in mice with an uptake mainly in the liver (Laakso et al., 1986).

Growth promoting implants using steroids have been used for over 40 years in livestock production (Preston, 1999). However, parenteral devices for systemic extended release of peptide and/or protein are continuously under development in veterinary medicine. One of the limiting factors is the cost of existing biodegradable polymeric systems which is not compatible with veterinary use. However, Contramid® is a very cost efficient polymer drug delivery system. Growth hormone (GH) secretion and insulin-like growth factor-I (IGF-I) plasma concentrations are closely related to growth performance, body composition and milk production in farmed animals (Dubreuil et al., 1990; Klindt et al., 1995, 1998; Foster, 1999). The pulsatile pattern of pituitary GH secretion results from the interaction of three hypothalamic factors (Tannenbaum, 1990; Kojima et al., 1999). Two are stimulatory: growth hormone-releasing factor (GRF) and Ghrelin. The third is inhibitory: somatostatin. Growth hormone acts on adipose tissue stimulating lipolysis and increases the production of IGF-I which stimulates protein synthesis (Corpas et al., 1993a). Twice daily subcutaneous injections (Corpas et al., 1992) and continuous infusion (Corpas et al., 1993b) of the human GRF over 2 weeks to elderly humans reversed age-related GH and IGF-I decreases and restored subnormal GH secretion and IGF-I concentrations, respectively. Moreover, somatotroph cell desensitization or depletion did not occur with continuous intravenous GRF infusion in humans over the same period of time (Vance et al., 1989) and in pigs over 6 days (Dubreuil et al., 1991). A GRF controlled delivery system should enhance GH secretion peaks during the non refractory responsive periods (Dubreuil et al., 1987) and may have a great interest in domestic animal production.

It is likely that Contramid® is a biocompatible polymer when implanted and can be administered parenterally for systemic or local extended release of a drug. The objectives of this study were, (1) to evaluate local host reaction when Contramid® is subcutaneously implanted in an animal model over a 180 day period; and (2) to determine the capacity of GRF analog loaded Contramid® pellets at regulating the release of this active peptide in growing pigs.

2. Materials and methods

2.1. Preparation of pellets

2.1.1. Mouse pellet

For assessment of Contramid® biotolerance in mice, dry blends of Contramid® with 16% w/w of hydroxypropylmethylcellulose (HPMC) were compressed using a 3 mm round punch providing 3 mm thick 25 mg placebo pellets.

2.1.2. Pig pellet

A potent analog of the natural human growth hormone-releasing factor, the hexenoyl-trans-3-hGRF(1–44)NH_2 (Hex-hGRF, Theratechnologies Inc., Quebec, Canada) was used. Three formulations were prepared by compression for solid implant manufacturing: the first formulation was 15 mg of pure Hex-hGRF peptide; the second and third formulations were dry blends of Contramid®/HPMC (84/16 w/w) with 30 and 15% w/w of Hex-hGRF, respectively. All pellets weighed 15 mg and measured 3 mm in diameter.

2.2. Surgical procedure in mice

Seventy 20–25 g, 8-week-old CD1 mice were used to evaluate the host reaction to subcutaneous implantation of Contramid®. The mice were randomly allotted in seven groups of ten animals which corresponded to the implantation time periods of 3, 7, 14, 30, 60, 120 and 180 days. Mice were anesthetized with intra-peritoneal administration of 2.5 mg ketamine hydrochloride (Rogarsetic, Rogar/STB Inc., Ont., Canada) and 0.25 mg xylazine (Rompun, Bayer Inc., Ont., Canada) simultaneously. The dorsal area of each animal was aseptically prepared for surgery by shaving and scrubbing with povidone iodine (Iodovet, Rougier Inc., Quebec, Canada). A 5 mm lumbar midline skin incision was performed followed by a 1 cm subcutaneous (SC) cranially directed tunnel. One placebo Contramid® pellet was implanted and skin was then sutured with a 4–0 non absorbable monofilament nylon (Ethilon™, Ethicon Inc., Ont., Canada). The animals were placed in individual cages, fed a normal diet and clinically monitored. Animals were euthanized by carbon dioxide asphyxiation. Postmortem examinations were limited to implantation sites. Lumbodorsal skin was resected, implantation sites were located and exposed for macroscopic examination. The implant with surrounding tissue was excised en bloc from mice and fixed in 10% buffered formalin solution for 24 h. The specimens were embedded in paraffin and 4 μm thick sections were performed.

2.3. Histologic examination

Histologic sections were stained with hematoxylin–phloxine–saffron (HPS). Periodic acid–Schiff (PAS) stain was also performed on two animals of each group. The slides were examined by two independent observers (CD, CG). The type of inflammatory cells and the presence of a fibrous capsule were evaluated. The density of inflammatory reaction was graded as follows, 0, no infiltrate; +, minimal infiltrate; ++, moderate infiltrate; and +++, marked infiltrate (Gomel et al., 1980; Neff et al., 1985).

2.4. Implantation in pigs

Thirty growing pigs weighing 50 ± 4 kg were randomly allotted into four treatment groups. In three groups ($n = 8$), animals were subcutaneously implanted at the level of the thoracic area near the elbow 1, 4 or 8 pellets with a 10 G trocar. All animals were administered a total dose of 15 mg GRF and the number of implants per animal depended on the payload, one implant of pure Hex-hGRF in the first group ($n = 8$); four 30% Hex-hGRF loaded Contramid® implants in the second group ($n = 8$); and eight 15% Hex-hGRF loaded Contramid® implants in the third group ($n = 8$). In a fourth group, six pigs were injected subcutaneously (10 μg/kg Hex-hGRF), every 12 h over 5 consecutive days and served as a positive control. Animals were individually kept in pens of 1.5×2.5 m and fed ad libitum. Daily clinical monitoring was performed until the end of the study. Venous blood samples were taken by venipuncture on days −1, 0, 2, 4, 7, 10, 14, 18, 22, 26 and 31 in groups 1, 2 and 3, and on days 0, 3, 6 and 9 in group 4. Serum IGF-I concentrations were determined by radioimmunoassay (Abribat et al., 1990).

All animal procedures performed in the present study were previously approved by the animal welfare ethic committee of the Faculty of Veterinary Medicine, University of Montreal.

2.5. Statistical analysis

Data generated in pigs (groups 1, 2 and 3) were analyzed using repeated-measures analysis of variance (ANOVA) with treatments and time as main factors. Separate one way ANOVA with Tukey's post hoc contrasts were used to test treatment pair's differences at each time point. A $P < 0.05$ was considered significant.

3. Results

3.1. Host response to Contramid®

Neither mortality nor general behavior modification was observed in mice. A 5 mm diameter

area of subcutaneous edema appeared transiently on the first 5 days post-implantation around the implant. Wound drainage was observed in five animals (7%) of different groups 1 week after implantation and the implant was always observed in place at post-mortem examination.

On macroscopic examination, Contramid® implants appeared as swelled pellets on days 3 and 7, then as gels on days 14 and 30. A thin fibrous capsule developed later and a small fibrous nodule eventually took place on day 180. Inflammatory reaction was always strictly limited to the implantation site without extension to surrounding tissues. Reaction diameter including the implant decreased from 5 to 2 mm from day 3 to 180, respectively. Mild hyperemia was observed close to the implant until day 60 in some animals. On day 3, inflammatory response was less intense around the implant than around the skin incision site (Fig. 1).

Recorded histologic grade score is reported in Table 1 for each group. Host reaction was similar in every animal. The predominant inflammatory cells associated to implanted Contramid® were polymorphonuclear cells (PMNs) and macrophages (Fig. 2). PMNs were mainly found in small groups inside the pellets. Inversely macrophages only surrounded the implant. From day 14 to 180, macrophage cytoplasm was spumous. Small number of lymphocytes were always observed while a few giant cells appeared only on day 180.

Neovascularization and fibrous septa were observed within the implant on days 7 and 14, respectively. Fibrovascular organization developed gently over time resulting in a complete fragmentation of the pellet (Fig. 3). A loose peripheral well-vascularized connective tissue developed around implants.

PAS stains specifically polysaccharides magenta including Contramid®. Contramid® and polysaccharidic by-products were always detected on PAS sections until day 180 (Fig. 3) and eventually in macrophage cytoplasm (Fig. 4). However, the total amount of Contramid® at the implantation site decreased and the macrophage crown shrunk over time.

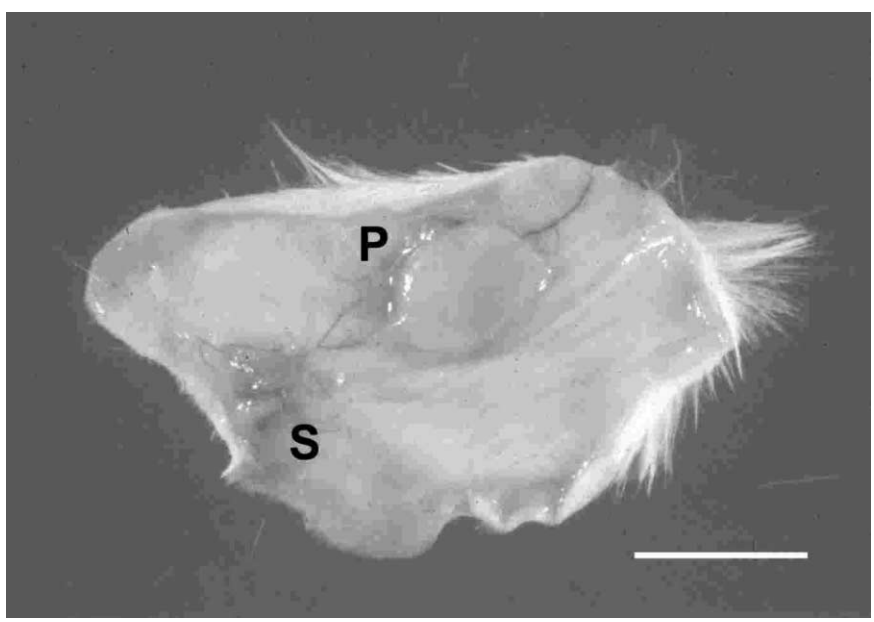

Fig. 1. Subcutaneous tissue. Mouse. Swollen Contramid® pellet (P) 3 days after implantation. Note the inflammation associated with the suture (S) (bar, 1 cm).

Table 1
Description of histologic reaction grade score to subcutaneous implanted Contramid® in mice

Days	PMNs	Macrophages	Giant cells	Lymphocytes
3	+++	+/++	0	0/+
7	+++	+++	0	+/++
14	++/+++	+++	0	+/++
30	+/++	+++	0	0/+
60	0	+++	0	0/+
120	0	+++	0	+/++
180	0	+++	0/+	+/++

PMNs, polymorphonuclear cells.

3.2. Hex-hGRF delivery in pigs

No animal exhibited pain, discomfort or local adverse reaction following implantation. Means of relative serum IGF-I concentrations compared with their respective initial levels (days −1 and 0) are shown in Fig. 5. The IGF-I concentrations were increased over a period of 10–14 days in all implanted groups and returned to baseline thereafter. In the pure Hex-hGRF implant group, IGF-I increased to the same extent as in the injection group on day 6 with a 2-fold increased concentration. Inversely burst was avoided for both Contramid® groups with a 1.5-fold increase plateau.

The three implanted groups had IGF-I concentrations significantly different ($P = 0.038$). Significant time effect and treatment–time interaction were observed ($P < 0.05$). Tukey's contrast test revealed a statistical difference ($P = 0.033$) between pure Hex-hGRF and Contramid®/Hex-hGRF (70/30) treatments, irrespective of the sampling day. However, when performed at each time point, contrast test revealed pure Hex-hGRF implant treatment to be significantly different ($P < 0.05$) to both Contramid®/Hex-hGRF treatments on days 4, 7, and 10 while significant differences between the two Contramid® treatments were not detected ($P > 0.05$). Significant time effect and treatment–time interaction were also observed ($P < 0.05$).

4. Discussion

This work reports for the first time the biocompatibility of Contramid®. The study was performed in mice because this animal model facilitates evaluation of a local reaction, pellet explantation and histologic examination over a period of 6 months. In pigs, local host response monitoring and implant retrieval would have been more difficult and less reliable. However, the subsequent Hex-hGRF delivery study was performed in a larger animal model like pigs in order to manufacture implants of an appropriate size and to use an adequate blood sampling procedure for IGF-I assay. Moreover, a potential application of such an implant in pigs is the improvement of carcass quality (Dubreuil et al., 1990; Klindt et al., 1995).

A 5 mm edema was always observed in mice at the implantation site over the first week. This was likely caused by the swelling of the polymeric matrix. Other clinical signs of inflammation (i.e. redness, heat and pain; Tizard, 1996) were indeed never observed. Contramid® is a hydrophilic polysaccharide characterized by an axial and radial swelling (Moussa and Cartilier, 1996). Tablet expansion occurs quickly in the early stages of hydration (Moussa et al., 1998). This process is believed to start immediately after the implantation procedure and is likely to be slower in vivo than in vitro due to the limited availability of

fluid. Wound drainage which occurred in some mice was probably more a complication of the surgical intervention instead of the host response to the polymer itself. Indeed macroscopic and microscopic observations of the corresponding implants in these animals were not different from other animals.

These results demonstrate an excellent biocompatibility of Contramid® implants. Similarly starch microspheres have been shown to be biocompatible when administered nasally in rabbits (Björk et al., 1991). Since they are degraded by serum amylase, starch microspheres were also used successfully and without toxicity in combination with a chemotherapeutic agent in humans by hepatic arterial injection in order to create a transient embolization (Dakhil et al., 1982). Neither toxicity nor morphological changes were observed when macrophages were exposed to starch microparticles in vitro (Artursson et al., 1987a).

It is of paramount importance to determine the type, duration and magnitude of cellular response in order to ascertain the biocompatibility of a material. Usual sequence of local reaction events after implantation of a biomaterial are acute then chronic inflammation followed by granulation tissue, foreign body reaction and then fibrosis (Anderson, 1994). Acute and chronic inflammatory responses are characterized by the predominance of short lifetime neutrophils and long lifetime macrophages, respectively. The temporal sequence of microscopic events observed in mice has demonstrated that Contramid® is a biocompatible material. However, intensity and time duration of previously described reaction steps may vary depending on the implant tested (Anderson, 1994).

Macrophages were a key component of the response to implanted Contramid®. It was the predominant inflammatory cell type observed from day 7 to 180 with a marked but local accumulation to the implantation site. It is known that macrophages may settle at the interface of implants for extended periods of time (Anderson and Miller, 1984). Furthermore it has been established that macrophages are stimulated by microparticulate polysaccharides, including starch but at a lesser extent (Artursson et al., 1987a). However, starch has no effect on macrophage nonspecific defense response such as cytotoxic activity (Artursson et al., 1987b). In the present

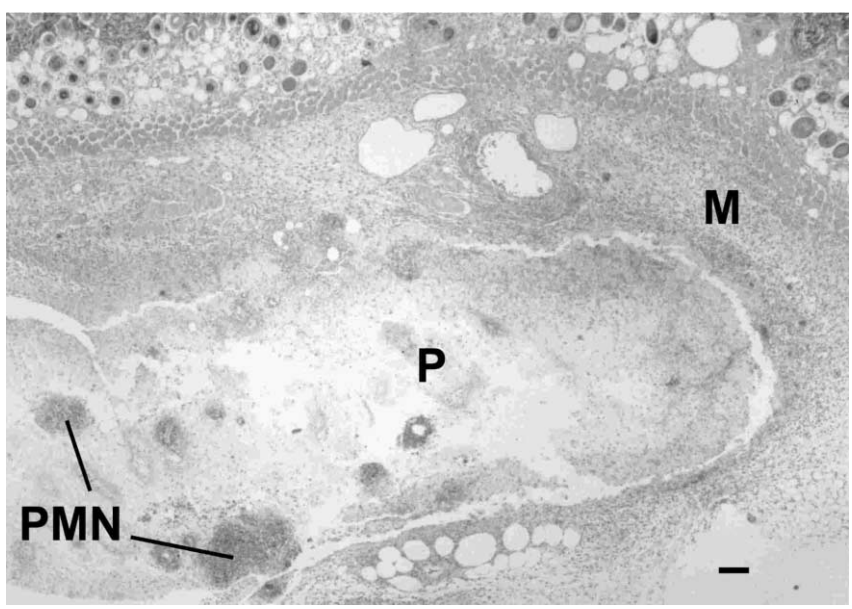

Fig. 2. Subcutaneous tissue. Mouse. Microscopic aspect of Contramid® pellet at day 7. The pellet (P) is infiltrated by PMNs and surrounded by macrophages (M). HPS stain (bar, 0.1 μm).

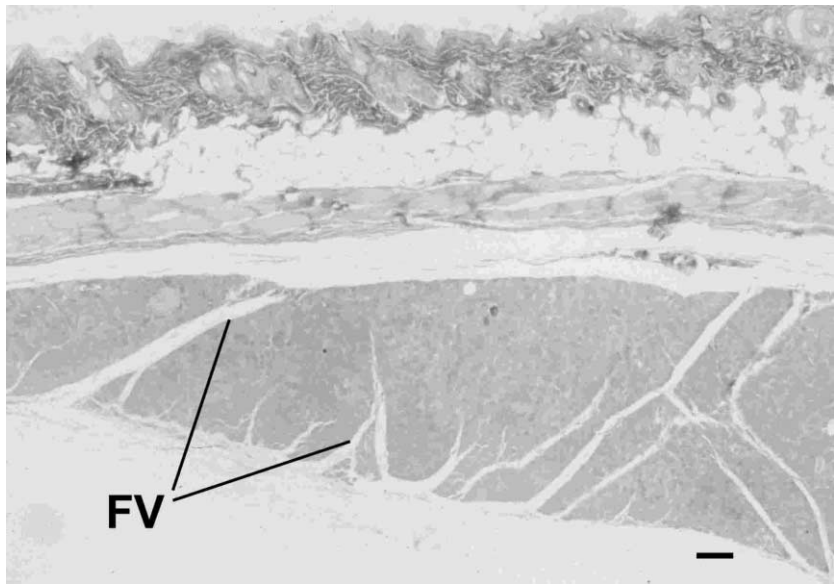

Fig. 3. Subcutaneous tissue. Mouse. Presence of PAS positive material at the implantation site (day 180) with fibrovascular septa (FV) and thin peripheral fibrous tissue. PAS stain (bar, 0.1 μm).

study, spumous macrophage cytoplasm from day 14 denoted a phagocytic activity. In addition, intracellular accumulation of PAS positive material was observed and the macrophage crown surrounding the implant as well as the amount of polymer were decreasing over time. In view of these findings, it can be concluded that Contramid® was degraded and eventually phagocytosed. An initial hydrolytic degradation of Contramid® is conceivable. Starch microparticles are degraded by serum α-amylase (Forsberg, 1978) and recently an enzymatic degradation of Contramid® tablets with α-amylase was reported (Rahmouni et al., 2001). Then Contramid® is phagocytosed by macrophages (Artursson et al., 1987a) and dissolved presumably by the action of α-glucosidases in the lysosomal compartment (Artursson et al., 1984). However, Contramid® absorption is only a hypothesis and should be verified in further studies.

Healing appeared as soon as 1 week after implantation of Contramid® with the proliferation of thin intra-implant fibrovascular septa. The foreign-body reaction is usually determined by the form and topography of the surface of the implant (Anderson, 1994). With Contramid®, the foreign-body reaction was mainly composed of macrophages. A few giant cells appeared on day 180 and the connective tissue around pellets was always well-vascularized and loose. Contramid® pellets obtained by powder compaction are porous and show a high surface to volume ratio. A high polymer porosity usually induces high ratios of macrophages and low fibrosis (Mohanty et al., 1992; Anderson, 1994). If this porosity is optimal it may allow a better vascular invasion of the polymer (Salzmann et al., 1997; Sharkawy et al., 1998). Mild fibrosis and neovascularization with Contramid® would enable a relatively efficient drug diffusion (Sharkawy et al., 1997) and plasma-tissue exchange (Sharkawy et al., 1998), respectively. The transport barrier between Contramid® implant and host appears to be limited.

Biofunctionality of Contramid® as a parenteral drug delivery device has also been demonstrated in the present study. Indeed for the first time an active peptide has been released from Contramid® pellets implanted subcutaneously. Absence of clinical host reaction and in vivo active peptide release with Contramid® implants are in agreement with the good biotolerance and the restrictive fibrosis previously observed in mice.

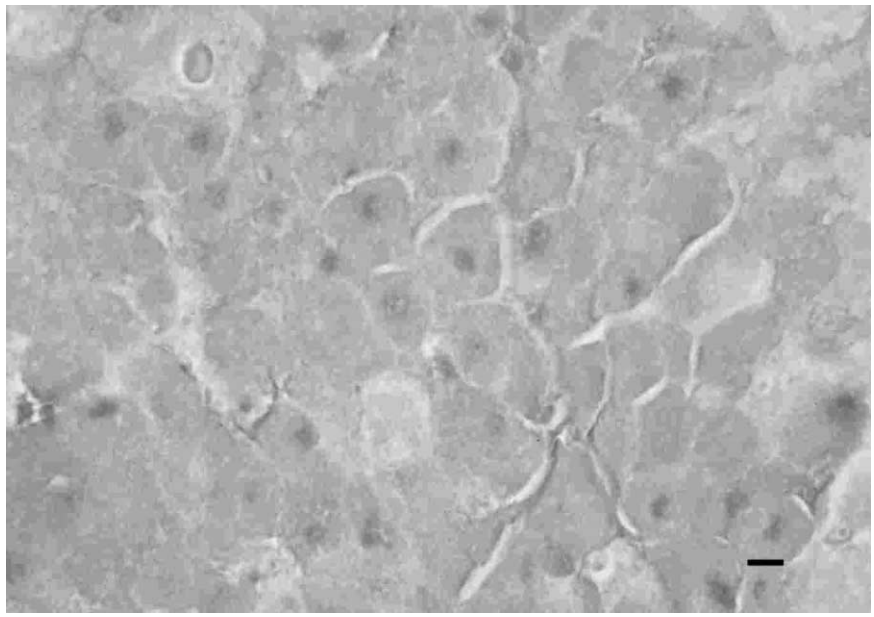

Fig. 4. Subcutaneous tissue. Mouse. PAS positive material localized in spumous cytoplasm of macrophages at day 180. PAS stain (bar, 0.05 μm).

A significant time effect on IGF-I concentrations for both Contramid® treatments reflected an exogenous GRF action at the pitituary level via a GH stimulation. Growth hormone directly or indirectly through IGF-I decreases lipogenesis and promotes protein accretion resulting in enhanced growth in food producing animals such as swine (Dubreuil et al., 1990). Efficiency of gain through decreased feed consumption but no increased rate of gain has been observed in pigs with subcutaneous administration of porcine-GH loaded implants (Klindt et al., 1995). Exogenous delivery profile of GH has been shown to have its biological importance. A GH pulsatile release device with an adequate frequency to achieve the physiological GH release as produced by the pituitary gland might be expensive and burdensome to develop for animal production application. However, long-term injection of a potent analog of GRF increased average daily gain, reduced food intake and improved feeding efficiency in swine (Dubreuil et al., 1990). A continuous administration of GRF over an extended period of time is likely to be more appropriate since GH physiological cycle is preserved. Indeed under these conditions, an increased GH peak amplitude has been reported in pigs (Dubreuil et al., 1991) as well as an increased GH peak frequency in humans (Vance et al., 1989; Corpas et al., 1993b) both

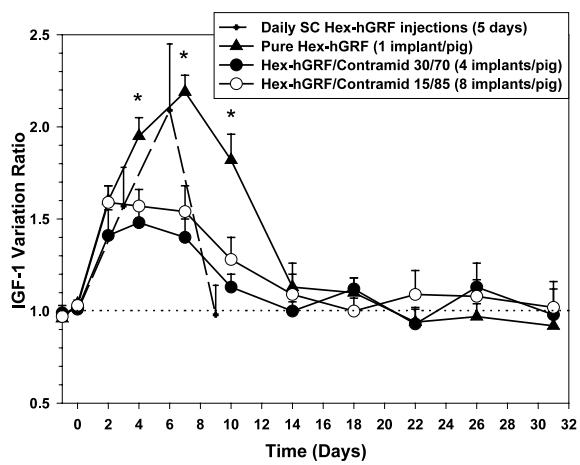

Fig. 5. Comparative effect on IGF-I secretion of three different Hex-hGRF (15 mg) implant formulations used in eight pigs per group and one solution injected (10 μg/kg BID for 5 days) in six pigs (*, $P < 0.05$). The average of initial IGF-I level is represented by the dotted line (……).

resulting in an increase of GH secretion and IGF-I concentration during GRF treatment.

IGF-I concentration profiles over time were not statistically different between the two Contramid® groups. Thus, Contramid® is a polymeric carrier characterized in vivo by a good robustness to Hex-hGRF loading level. Inversely PLGA microspheres administered subcutaneously to swine were characterized by a definite response depending on loading level of an analog of natural porcine GRF (pGRF) (Thompson et al., 1997).

The present study demonstrates that initial burst as observed with pure Hex-hGRF implants has been significantly controlled with addition of Contramid® providing a safe IGF-I concentration plateau. However Hex-hGRF activity time of 10 days on IGF-I concentration was not extended with Contramid® implants since no significant difference was detected among the three implant treatments on day 14 and no recurrent IGF-I increase was observed thereafter. All animals were given the same dose of Hex-hGRF (15 mg) but its bioavailability estimated via IGF-I area under the curve is different between pure Hex-hGRF and Contramid® treatments. Since active period was similar for all groups it is, therefore, hypothesized that in vivo some Hex-hGRF was lost in the Contramid® implants due to instability of the peptide. Similarly neither the dose nor the loading level of an analog of pGRF in PLGA microsphere formulation had an effect on the duration of response in swine where the observed activity also lasted only 9–12 days (Thompson et al., 1997).

5. Conclusions

For the first time Contramid® implants were tested in vivo. It was demonstrated that this polymer is well tolerated with an excellent biocompatibility. Macroscopic examination following implantation revealed a mild inflammatory reaction always strictly limited to the implantation site. Microscopically, it has been shown that Contramid® is degraded and eventually phagocytosed by macrophages. Development of a mild peripheral fibrosis and an intra-implant neovascularization suggested firstly a limited transport barrier between implant and host and secondly a possible diffusion of a drug from the polymer matrix, respectively.

This study also demonstrates the in vivo diffusion of an active peptide from Contramid® matrix. Contramid® biofunctionality as a systemic drug delivery device after implantation is, therefore, established. During all the Hex-hGRF activity period, the delivery was controlled with Contramid® enabling increased IGF-I concentrations within a safe range which has been reported to improve carcass quality in meat animals. The Contramid® implant concept could be appropriate for this purpose but the issue of Hex-hGRF stability issue has to be resolved first.

More generally, Contramid® implantation concept may be used for various site-specific applications in the future. Such drug delivery systems have several advantages compared with conventional drug therapies (Langer, 1990), (1) absence of drug concentration peaks and throughs; (2) drug delivery to a particular body compartment at an adequate concentration; (3) protection for drugs rapidly degraded; (4) increase of drug efficacy and patient compliance; and (5) decrease of treatment cost.

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