Controlled release of dexamethasone from subcutaneously-implanted biosensors in pigs: Localized anti-inflammatory benefit without systemic effects

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Abstract: Chronically implanted biosensors typically lose sensitivity 1–2 months after implantation, due in large part to the development of a collagen-rich capsule that prevents analytes of interest from reaching the biosensor. Corticosteroids are likely candidates for reducing collagen deposition but these compounds have many serious side effects when given over a prolonged period. One method of assessing whether or not locally released corticosteroids have a systemic effect is to measure cortisol concentrations in venous serum. We hypothesized that a very low release rate of the potent corticosteroid, dexamethasone, would lead to a localized anti-inflammatory effect without systemic effects. We found that reduction in subcutaneous granulocytes (primarily eosinophils), and to a lesser extent, reduction of macrophages served as a good local indicator of the steroid effect. When released over a 28-day period, a total dexamethasone dose of $\leq$0.1 mg/kg led to a consistent reduction in the number of granulocytes and macrophages found in the local vicinity of the implant without a reduction of these cells at distant tissue locations. The lack of suppression of serum cortisol with these doses confirmed that low-release rates of dexamethasone can lead to consistent local anti-inflammatory effects without distant, systemic effects. © 2010 Wiley Periodicals, Inc. J Biomed Mater Res 94A: 280–287, 2010

Key words: dexamethasone; foreign body response; medical device; eosinophil; glucose sensor

INTRODUCTION

In long term studies in dogs[1] and in rats[2] in which subcutaneous glucose sensors were fully implanted, we found that sensor function was most accurate and reliable during the first 4–6 weeks. After implantation, foreign body capsules formed around the sensors. Over time, these capsules became progressively enriched with collagen, and the sensors grew progressively less sensitive to changes in glucose. Reichert and coworkers have clearly shown that over time, small molecule analytes such as glucose are progressively less able to diffuse through the foreign body capsule.[3–5] Taken together, these studies suggest that deposition of collagen and other extracellular matrix components eventually leads to loss of the ability of analytes to permeate into the sensing layers of biosensors.

Several groups have become interested in slow, localized release of corticosteroids in an effort to reduce the foreign body fibrosis associated with subcutaneously-implanted devices. Although there have been several promising reports,[6,7] there are many severe side effects of corticosteroids, which include elevated blood glucose, elevated blood pressure, development of fat deposits in certain regions of the body, and ocular complications.[8]

It is well known that even modest elevations of serum corticosteroid levels will consistently suppress endogenous production of cortisol.[9] For this reason, we elected to monitor peripheral serum levels of cortisol to determine a dose of dexamethasone that would exert a favorable local effect on the foreign body response without causing systemic suppression of cortisol production. We carried out a dose-ranging study during which dexamethasone was continuously delivered to the tissue interface of subcutaneously implanted (mock) glucose sensors via a miniature osmotically driven pump.

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Another goal of this study was to conduct a histologic assessment of the subcutaneous tissue near the source of the corticosteroid infusion. We hypothesized that if the infusion rate of dexamethasone was kept very low, we would observe an anti-inflammatory effect localized near the delivery site without histologic changes distant from the delivery site.

METHODS

Biosensor fabrication, animals, and surgical procedures

This investigation was a dose-finding study of a controlled-release corticosteroid drug, dexamethasone (DEX). The goal was to find a dose, given slowly by the subcutaneous route over 28 days via miniature osmotic pumps that would cause a local effect while avoiding systemic effects. Systemic effects were assessed by measuring the degree of suppression by DEX of the hypothalamic-pituitary-adrenal axis. Pigs were chosen because of the similarity of their skin and subcutaneous tissue to those of humans. These Yucatan mini-pigs had grown to large adult size and had a body weight of 60–70 kg. Histologic studies were carried out on subcutaneous tissue surrounding mock biosensors that had been implanted for 28 days.

The mock biosensor arrays contained the same materials as functional glucose sensors, except for the absence of electronic circuitry. The dimensions of each biosensor were 53 mm × 33 mm × 9 mm. On the surface of each biosensor was a single disk-shaped central reference electrode made of silver, whose diameter was 13 mm and which was chloridized by exposure to FeCl.[1] The central reference electrode was surrounded by four platinum indicating electrodes also mounted on the sensor surface (the devices are termed "arrays" because of the multiple indicating electrodes, each of which serves as an independent sensor when fitted with electronics). The exposed dimensions of these indicating electrodes were 10 mm × 0.5 mm. Sensors were coated with an amphipathic polyurethane that contains polyethylene oxide (PEO) moieties. In previous work, this biocompatible polyurethane with PEO led to a less intense foreign body response when compared with a polyurethane without PEO.[10] The amphipathic nature of the polyurethane is necessary to allow both oxygen and glucose to pass to underlying layers. The polyurethane coating is the only material in contact with subcutaneous tissue.

Tissue was harvested for histologic analysis from four locations: (1) tissue located very close to the DEX source, the tissue being obtained from one sensor array at a location 2 mm away from the source of DEX; (2) tissue obtained further from the DEX source, in the same sensor array, 17 mm away from the source of DEX; (3) tissue obtained very far away from the DEX source, 300–400 mm away from the source of DEX. In this condition, the tissue was obtained from a site overlying another sensor array that was implanted at a location distant from the array containing the DEX source. For this distant sensor array, saline (not DEX) was infused from an attached osmotic pump; and (4) tissue obtained from an animal in which no DEX was released. In this pig, saline was also released from the sensor array by an attached osmotic pump.

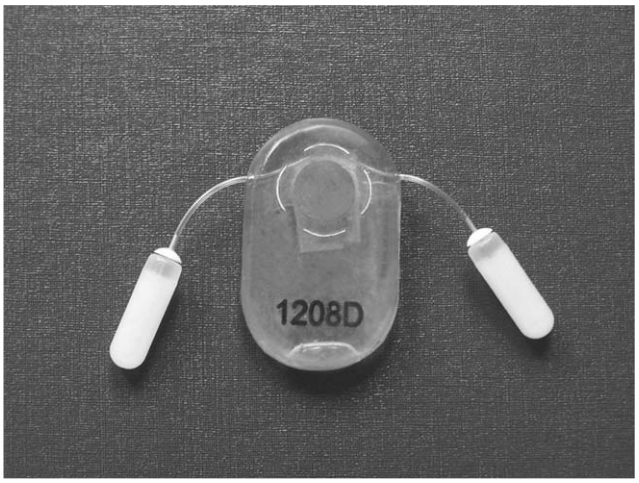

Figure 1. Biosensor array with two osmotic pumps, the tubing for which enters the central cavity then terminates on the sensor face. The dimensions of the biosensor are 53 mm × 33 mm × 9 mm. On the surface of each biosensor is a single common reference electrode made of silver, whose diameter is 13 mm. The reference electrode is surrounded by four platinum indicating electrodes, also mounted on the surface. For lower release rates of dexamethasone, a single osmotic pump was used.

The normal saline or DEX was released from the surface of the sensor array slowly over 28 days via a mini-osmotic pump (Alzet pump, Durect, Cupertino CA). The DEX (Sigma chemicals, No. D1756) was dissolved in (2-Hydroxypropyl)-β-cyclodextrin (Sigma H5784). Dexamethasone dosing was administered according to the animal's body weight and is thus expressed as mg per kg body weight. The drug delivery tube from the osmotic pump(s) was routed into the central cavity of the sensor array then upto the surface of the array so that the end of the tube terminated on the sensor surface, allowing DEX or saline to be released into the subcutaneous tissue overlying the array. If the doses were larger, two pumps were required in which case the surface tube outlets were immediately adjacent to one another. The pump capacities were either 200 μL (pumping rate 0.25 μL per hr) or 2 mL (pumping rate 2.5 μL per hour).

Figure 1 shows a photograph of two osmotic pumps attached to a sensor array. All pumps were aseptically prepared and incubated while attached to the sensor at 37°C in normal saline for 16 h before implantation, to prime the pumps. At the end of the study, each pump was opened to inspect the contents. In all cases, there was a very small amount of DEX that remained, verifying that almost all of the DEX had been released.

Each mature male Yucatan mini pig had 3–4 sensor arrays implanted subcutaneously in the dorsal region, one of which released DEX. The other 2–3 were placed 300–400 mm from the DEX-releasing arrays and releases saline. Sensor arrays were oriented with the electrodes facing away from the skin, toward the fascia and muscle.

Arrays, including about 1 cm of surrounding tissue, were removed 28 days after implantation. During explantation, tissue was collected from the sites that were described earlier. For orientation purposes, the surface of the tissue against the biosensor was carefully marked with an indelible tissue ink (Mark-It, Thermo Fisher Scientific, Waltham MA) during the explant procedure and then fixed in 10% zinc formalin for later paraffin sectioning. Marking the tissue allowed for the orientation of the tissue to be preserved throughout the fixation and embedding process. Implant and explant procedures were carried out during inhalation anesthesia with 2% isoflurane (after induction with telazol). Animals were not euthanized at the end of the study and were available for additional studies after the explant wounds healed and after at least 2 weeks had elapsed. Six pigs were used in this study. Animals were housed at the Legacy Clinical Research and Technology Center in Portland, OR, an AALAC-approved site. All procedures were approved by the Legacy Institutional Animal Care and Use Committee before initiation of the study.

Histology and counting of granulocytes and macrophages

Formalin-fixed foreign body capsule tissue samples were processed and embedded in paraffin. Sections that were 5–7 μm thick were cut with a microtome followed by staining with hematoxylin and eosin. Other sections were stained with Masson's trichrome to identify collagen. All image acquisition was performed using a Retiga 1300 and QCapture (QImaging) on a Leica DMR upright microscope (Wetzlar, Germany). To carry out cell counts, for each study condition, microscopic fields (each of which was 100 μm × 100 μm), obtained at the sensor-tissue interface marked by black indelible ink, were randomly chosen from an H and E-stained section.

The number of granulocytes was counted in this field by two observers who were blinded to the study condition. The mean values from the two observers were used for analysis. Field selection and cell counting were done using NIH ImageJ. The vast majority of the granulocytes were eosinophils; a smaller number were neutrophils.

In addition, two observers blinded to the study condition also counted macrophages in the same fashion. Macrophages have distinctive pale oval nuclei with dark nucleoli. They are easily distinguished from fibroblasts (which are spindle-shaped) and from eosinophils.

Cortisol measurements

Serum cortisol was used as an index of hypothalamic-pituitary-adrenal axis suppression given the fact that as the axis is suppressed by exogenous steroids, the adrenal gland reduces its endogenous production of cortisol.[11] On the morning of the mock biosensor implant or explant, during isoflurane anesthesia, a blood sample was withdrawn from a subclavian vein for measurement of a serum cortisol level. Serum was prepared by allowing blood to clot for 15–20 min then centrifuging at 2500 rpm for 10 min. The sample was stored at 4°C until ELISA assay by a commercial laboratory service (Idexx Laboratories, West Sacramento, CA).

Data analysis

For differences in data values when comparing one animal to another, unpaired, two-tailed *t*-tests were used. For differences in data values in comparing results from one animal at two time points, paired two-tailed *t*-tests were used (e.g., cortisol at baseline vs. cortisol during DEX treatment in the same animal). Data were distributed primarily in a parametric fashion; for this reason, parametric statistics were used.

RESULTS

Suppression of serum cortisol values

Serum cortisol levels were measured at the time of implant to serve as a baseline level and at the time of the 28-day explant. Upon termination of the study, there was a very small amount of dexamethasone remaining in the pump indicating that the delivery of Dex was maintained throughout the length of the study.

The baseline pre-DEX morning cortisol values were in the 3–11 μg/dL range for all the animals (7.25 ± 1.1, mean ± SEM, $n = 8$). These values are somewhat lower than typical morning values in humans. The animals then received a range of DEX doses given over the 28-day period, as follows: no DEX ($n = 1$); 0.05 mg/kg ($n = 2$), 0.1 mg/kg ($n = 1$); 0.168 mg/kg ($n = 2$); 0.28 mg/kg ($n = 1$); or 0.7 mg/kg, ($n = 1$).

The cortisol values at the 28-day time point segregated into two groups: those whose cortisol values did not suppress (designated as no/low dose DEX: 0.1 mg/kg or lower) and those whose values did suppress (designated as high-dose DEX). Cortisol values in the no/low-dose DEX group averaged 5.15 ± 0.75 μg/dL, similar to baseline (NS). In contrast, cortisol values in the high-dose DEX group were markedly suppressed to a mean level of 0.6 ± 0.19 μg/dL ($p = 0.045$ vs. initial value and $p = 0.009$ vs. 28 day values for no/low-dose DEX). Cortisol values, plotted as a function of DEX dose, are shown in Figure 2.

Granulocyte counts

All results are reported as the mean of the number of granulocytes found in a 10,000 μm^2 area immediately adjacent to the sensor array. Most of the granu-

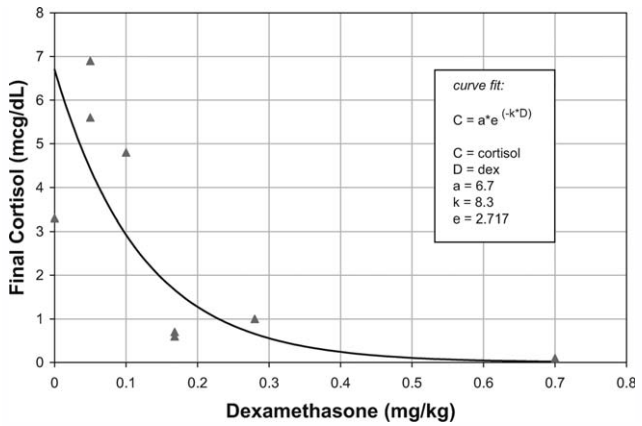

Figure 2. Serum cortisol levels 28 days after beginning continuous subcutaneous dexamethasone administration. Cortisol levels (y-axis) with respect to total dexamethasone dose over 28 days (x-axis) were fitted with the exponential decline equation as noted in the inset. Although the data can be grouped in a binary fashion according to low dose (0.1 mg/kg and below) versus high dose (over 0.1 mg/kg), curve-fitting was used here because the physiological suppression of cortisol production by the adrenal gland is thought to be a continuous function of circulating cortisol levels, not a discrete binary function. Irrespective of the grouping method, suppression of cortisol was seen only at total DEX doses of over 0.1 mg per kg.

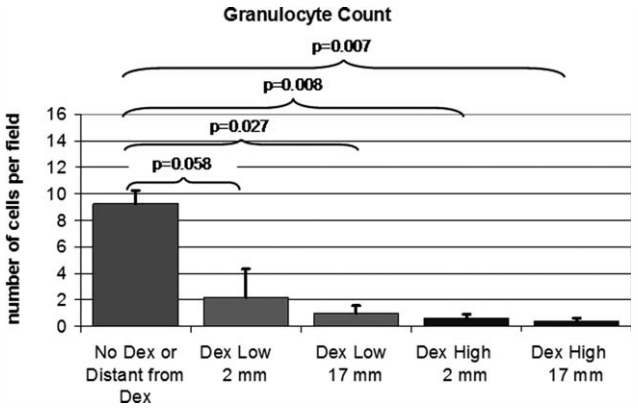

Figure 3. Granulocyte counts in SC tissue, according to distance from DEX and dose of DEX (mean ± SEM). Note that granulocyte count was a sensitive indicator of the DEX effect, and the effect to reduce cell count was noted even 17 mm away from the source of DEX. Because there was no difference in cell count in the No DEX condition versus cell count 300–400 mm from the DEX source, those data were merged. Each microscopic field was 100 × 100 μm. The number of fields was $n = 38$ for No DEX or distant; $n = 3$ for low dose, 2 mm; $n = 3$ for low dose, 17 mm; $n = 4$ for high dose, 2 mm; and $n = 4$ for high dose, 17 mm.

locytes were eosinophils. In the cases where saline was released, the mean number of granulocytes 2 mm from the saline source and 17 mm from the saline source were not significantly different, so data from these two sites were merged.

We found that delivery of DEX to the implant surface led to a marked reduction in the number of eosinophils found in the tissue adjacent to the implant (Fig. 3). This reduction was observed in tissue 2 mm away from the DEX delivery site as well as in tissue obtained 17 mm from the delivery site. As shown in Figure 3, the number of eosinophils found adjacent to the implant was markedly reduced with both low and high-DEX doses, compared with no DEX administration or in tissue distant (300 mm) from the implant. For number of microscopic fields, see Figure legends.

Figure 4 shows several representative H and E images, demonstrating the presence or absence of

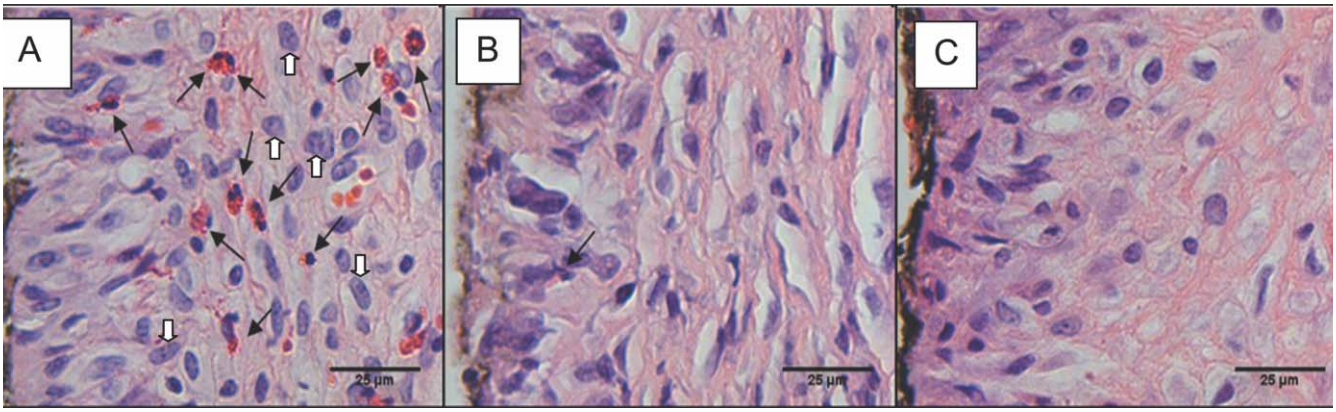

Figure 4. Representative images of H and E staining for (A) tissue with minimal to no exposure to DEX (obtained 300–400 mm from the DEX release site), (B) tissue with high exposure to DEX (obtained 2 mm from a low-dose dexamethasone release point), and (C) tissue with very high exposure to DEX (obtained 2 mm from a high-dose DEX release point). Note that the samples from the low-exposure site show the presence of numerous eosinophils (black arrows indicating cells with lobulated nuclei and acidophilic granules) and macrophages (white arrows indicating oval cells with pale nuclei). In contrast, samples obtained from the high-exposure sites contained very few eosinophils and a reduced number of macrophages. The scale bars represent 25 μm. For each panel, the location of the tissue-sensor interface is at the left.

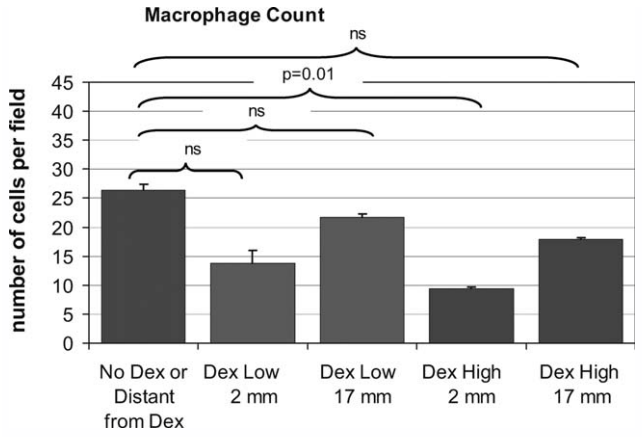

Figure 5. Macrophage count in tissue obtained at various distances from the DEX source and at different DEX doses. For the tissue exposed to the highest dose of DEX (2 mm from the source of DEX that led to systemic suppression of serum cortisol), there was a highly significant reduction in macrophage count. Macrophage count was not as sensitive an indicator of the DEX effect as was the granulocyte count. Data are expressed as mean ± SEM. Each microscopic field was 100 ×100 μm. Number of fields was n = 40 for No DEX or distant, n = 3 for low dose, 2 mm; n = 3 for low dose, 17 mm; n = 4 for high dose, 2 mm; and n = 4 for high dose, 17 mm.

granulocytes and macrophages, according to study condition. (Mast cells, which also contain acidophilic granules, can be mistaken for eosinophils, but we believe that the vast majority of the granular cells in these sections were eosinophils, given their bi-lobed appearance. In contrast, mast cells have a single, round, nonlobar nucleus.)

Macrophages

As shown in Figure 5, there was a trend for the macrophage count to be reduced as proximity to the source of DEX and dose of DEX were increased. The macrophage count 2 mm from high-dose DEX was significantly lower than the count in the No DEX tissue and in tissue obtained 300–400 mm from the DEX source. Macrophage counts in other DEX conditions, including the low-dose DEX and the tissue 17 mm from the DEX source, were not significantly different from No DEX or 300–400 mm from DEX source results. There were only a few multinucleated giant cells, seen in the No DEX or distant from DEX tissue.

General foreign body capsule observations

H and E stained sections were also used to observe overall tissue morphology and cellular organization. In-tissue was obtained from the No DEX sites or distant (300–400 mm) from the DEX source; the connective tissue was quite dense and very cellular at the interface with the biosensor. In addition, at these locations, the collagen generally had an organized, fibrous structure and resembled dense, irregular connective tissue. Interestingly, Masson's trichrome staining revealed that little to no collagen is located in the dense tissue immediately adjacent to (within 50–100 μm of) the biosensor. Instead, collagen is located further away (more "inland") from where biosensor was in contact with the tissue. In terms of orientation, the collagen was organized into fibers, which were parallel with the flat surface of the biosensor array. Trichrome staining also revealed that areas with reduced cellularity tend to have higher collagen content. The organization and the "inland" location of collagen in a tissue sample acquired far away from the DEX source is shown in Figure 6.

Irrespective of whether the SC tissue was acquired 2 or 17 mm from the DEX release source, the connective tissue tended to be less dense with fewer acute and chronic inflammatory cells when compared with tissue obtained 300–400 mm from the source. There was also a tendency for collagen in tissue near DEX delivery to have a patchy appearance.

DISCUSSION

The goal of this study was to find a corticosteroid release dose, released slowly over 28 days, that was sufficient to provide, localized anti-inflammatory protection to an implanted device and yet was low

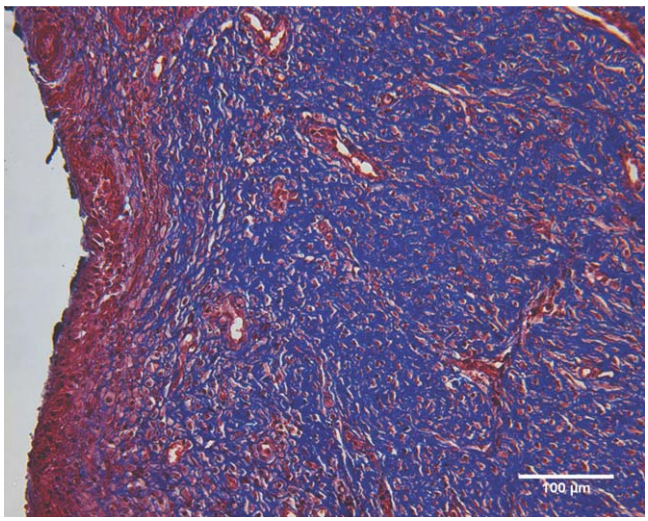

Figure 6. Subcutaneous field, stained with Masson's Trichrome, acquired along the implant interface but 300–400 mm away from DEX source. Location of the sensor array was on the left. Note that there is a band of tissue (whose width is usually 60–90 μm) with very little collagen immediately adjacent to the sensor interface. The region of dense collagen deposition (blue) is set back (further "inland") from this band.

enough to avoid systemic effects. We chose pigs for this study based on the generally accepted belief that dermal and subcutaneous tissues in pigs are similar to those of humans.[12,13] Our data suggest that a DEX dose of 0.05–0.10 mg/kg, given as a constant subcutaneous infusion over 28 days, leads to predictable local histologic effects and does not suppress systemic cortisol levels below baseline levels.

One of the functions of the hypothalamus and pituitary gland is to constantly monitor the circulating levels of corticosteroids. When such levels are supraphysiologic, endogenous corticotropin (ACTH) secretion by the pituitary is suppressed, thus leading to suppressed endogenous production of cortisol by the adrenal cortex. For this reason, decline of serum cortisol levels is a quite sensitive indicator of corticosteroid administration. For example, even with doses of prednisone that are considered to be rather low (e.g., 0.1 mg per kg per day in humans), circulating cortisol levels are suppressed.[9] In this study, we found a clear suppression of morning venous cortisol levels with DEX doses of at least 0.17 mg/kg over the 4 week period but found no suppression with doses of 0.1 mg/kg or less.

Even with low doses of DEX, we found consistent localized histologic effects, leading us to believe that the slow release DEX from the surface of a biosensor might well decrease the intensity of the foreign body response and thus promote long-term biosensor function. The most sensitive effect of DEX was a predictable, marked local reduction in granulocyte (mainly eosinophil) count. In fact, the suppressive effect of low-dose DEX on the granulocyte count was essentially the same as that seen in high-dose DEX and was as prominent in tissue 17 mm from the DEX source as it was in tissue 2 mm from the source. Low-dose DEX delivery did not appreciably diminish serum cortisol levels. Further, the number of granulocytes found in the tissue was similar to No DEX controls, also indicating the lack of a systemic corticosteroid effect. Eosinophils can be identified in H and E stained sections as cells with multiple acidophilic (red) granules in the cytoplasm. It is possible that some of the cells that we identified as eosinophils were in fact mast cells. Nonetheless, since corticosteroids would be expected to decrease both cell types, mistaking one for the other does not diminish that finding acidophilic granule-containing cells are an excellent metric of the steroid effect in subcutaneous foreign body capsule tissue.

The histologic findings and the lack of circulating cortisol suppression support the concept that the effect of DEX at a dose of 0.1 mg/kg or less is confined to a small localized area. For this reason, we would expect that analogous doses in humans would not cause adverse effects such as glucose intolerance, osteoporosis, hypertension, or central obesity. However, due to possible species differences, the comparable doses/release rates in humans might be different from those in pigs, even when adjusted according to body weight or body surface area. At any rate, the adjusted doses/release rates in this pig study might well be a good starting point for similar dose-ranging studies in humans.

We also found that there was a significant effect of DEX to reduce macrophage number in the subcutaneous tissue, though the magnitude of this effect was not as marked as the effect on eosinophil count reduction. An action of corticosteroids to suppress histiocytes (tissue monocytes or macrophages) has been previously reported,[14] though we are unaware of previous reports that specifically have addressed macrophages in foreign body capsule tissue.

Macrophages are not difficult to identify and have oval, pale nuclei with one or more prominent nuclear inclusions. Nonetheless, we acknowledge that in the absence of a specific stain for macrophages in this study, we could have misidentified some of the macrophages, which can on occasion be mistaken for fibroblasts (which generally stain darker and are more slender and spindle-shaped in a two-dimensional field). On the basis of the findings of this study, we believe that a reduction of eosinophil- and macrophage number in subcutaneous foreign body tissue is a convenient and sensitive method of assessing the steroid effect. Eosinophils are active in mediating immune responses to foreign invaders and can lead to autoimmune disturbances such as asthma that are characterized by tissue inflammation.[15] Impairment of macrophage number and/or function may also have favorable effects on the foreign body response. Macrophages are known to release a large number of cytokines that mediate many aspects of the foreign body response. One of these chemicals is transforming growth factor-β, which we found to be very overexpressed in the foreign body capsule (FBC) surrounding subcutaneous implants[16] and which leads to increased connective tissue growth factor, which is also markedly overexpressed in FBC tissue.[17]

It has been known for many decades that corticosteroids reduce fibrosis under many circumstances and in many tissues. For example, in 1969, it was shown that rabbits that received corticosteroids had delayed wound healing that persisted for weeks after corticosteroids were withdrawn.[18] In addition to such effects on wound healing, there are many adverse systemic effects such as hypertension, glucose intolerance/diabetes, central obesity, glaucoma, cataracts, osteoporosis, lipid abnormalities, and behavioral problems.[8] Despite these systemic risks, the local anti-fibrotic and anti-inflammatory action of corticosteroids can be quite beneficial when the

delivery rate is sufficiently low. For example, slow elution of dexamethasone from pacemaker electrodes has been very successful in avoiding the need to increase electrical stimulation over the long term. Furthermore, the dexamethasone release rate used in these pacemaker electrodes to avoid fibrosis is so low that a very small steroid reservoir can last for at least 10 years.[19]

Several groups, including ours, have become interested in slow, localized release of corticosteroids in an effort to reduce foreign body fibrosis that develops around implanted devices. This type of fibrosis, in which parallel bundles of collagen are formed in the extracellular matrix, is particularly detrimental to the long-term function of implanted biosensors that require inward diffusion of analytes such as glucose to sustain normal function. In several prior studies, we found that most fully implanted amperometric glucose sensors are capable of accurate function for only 4–6 weeks, after which time, glucose entry into the sensor is reduced, leading to reduced sensitivity, and eventual loss of function.[1,2] Histologic studies of the foreign body capsule found that porous membranes (or porous scaffolds)[3–5,10,20–22] and slow subcutaneous infusion of vascular growth factors[23] retarded foreign body fibrosis to some extent. Nonetheless, in our opinion, these improvements are unlikely to extend sensor lifetime beyond 6 months.

Moussy and coworkers have explored the effect of dexamethasone (DEX) on the foreign body response and found that DEX slowly released from microspheres had a beneficial effect on the foreign body response to subcutaneously-implanted sutures in rats.[24] In an attempt to understand the spatial effect of locally released steroids, they also found that the primary effect of DEX was confined to a very small area (several mm), as verified by direct *in vivo* measurement, mathematical modeling,[6] and autoradiography in subcutaneous tissue slices.[25] The Burgess laboratory has demonstrated the use of a PLGA microsphere-based controlled release system to release DEX in concert with angiogenic growth factors.[7,26,27] Norton et al. found that when DEX was coadministered with VEGF, there was a reduction in inflammation without the loss of vascularity that was seen with DEX alone.[28]

Our current results in pigs implanted with subcutaneous polyurethane-coated devices demonstrates that there are doses of DEX which exert a clear local effect without suppressing circulating cortisol levels and without causing histologic effects at distant sites. Our results, taken together with those of the Moussy, Burgess, and Reichert groups provide strong support for the prospect of avoiding deleterious systemic effects of corticosteroids while achieving beneficial local anti-inflammatory effects. Still needed are long-term studies that assess whether the functional life of steroid-releasing biosensors can be successfully prolonged by this strategy.

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