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Impact of macrophage deficiency and depletion on continuous glucose monitoring *in vivo*

Ulrike Klueh [a,b,*], Yi Qiao [a,b], Jackman T. Frailey [a,b], Donald L. Kreutzer [a,b]

[a] Center for Molecular Tissue Engineering, University of Connecticut, School of Medicine, Farmington, CT 06030, USA
[b] Department of Surgery, University of Connecticut, School of Medicine, Farmington, CT 06030, USA

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ABSTRACT

Although it is assumed that macrophages (MQ) have a major negative impact on continuous glucose monitoring (CGM), surprisingly there is no data in the literature to directly support or refute the role of MQ or related foreign body giant cells in the bio-fouling of glucose sensors *in vivo*. As such, we developed the hypothesis that MQ are key in controlling glucose sensor performance and CGM *in vivo* and MQ deficiencies or depletion would enhance CGM. To test this hypothesis we determined the presence/distribution of MQ at the sensor tissue interface over a 28-day time period using F4/80 antibody and immunohistochemical analysis. We also evaluated the impact of spontaneous MQ deficiency (*op/op* mice) and induced-transgenic MQ depletions (Diphtheria Toxin Receptor (DTR) mice) on sensor function and CGM utilizing our murine CGM system. The results of these studies demonstrated: 1) a time dependent increase in MQ accumulation (F4/80 positive cells) at the sensor tissue interface; and 2) MQ deficient mice and MQ depleted C57BL/6 mice demonstrated improved sensor performance (MARD) when compared to normal mice (C57BL/6). These studies directly demonstrate the importance of MQ in sensor function and CGM *in vivo*.

1. Introduction

The central role of monocyte related cells (MRC), i.e. macrophages (MQ), giant cells (GC) and dendritic cells (DC), as well as their products, in controlling tissue reactions associated with human disease, including foreign body reactions (FBR), is universally accepted. Although MQ are histologically associated with implantable biosensors *in vivo*, there is no direct *in vivo* evidence of the role for MQ or their products in the loss of sensor function seen *in vivo*. Filling this gap in our understanding would not only clarify the role of MRC and their products in the loss of sensor function *in vivo*, it will also help identify key targets for therapeutic intervention and sensor design in an effort to extend sensor lifespan *in vivo*.

To begin to fill this gap in our knowledge we propose to determine the contribution of monocyte/macrophages (M/MQ) to sensor performance *in vivo*. In order to demonstrate the direct contributions of M/MQ in controlling sensor function and CGM *in vivo*, we first characterized MQ accumulation at sensor–tissue interface at sensor implantation sites using immunohistochemical technology specific for MQ. We also evaluated CGM performance in mutant mice deficient in M/MQ (op/op mice) [1] and in mice depleted of M/MQ (Human Diphtheria Toxin Receptor (hDTR) knock-in mice) [2]. Normal littermates (op/op studies) or C57BL/6 (hDTR studies) mice were among the controls for these studies. The results of these studies demonstrated that 1) MQ accumulate at sensor–tissue interface during CGM ultimately forming a MQ barrier surrounding the implanted sensor *in vivo*; and 2) that either deficiency or depletion of M/MQ enhances CGM when compared to CGM in normal/control mice. Using these spontaneous and transgenic models clearly demonstrates the importance of MQ in sensor function and CGM, and underscores the need for future studies to understand and overcome negative impacts of MQ on CGM *in vivo*.

2. Materials and methods

2.1. Mouse models

All mice used in these studies where obtained from Jackson Laboratories, Bar Harbor Maine. These mice included *op/op* mice (B6; C3Fe a/a-Csf1op/J, Jax Stock # 00231), DTR mice (B6.FVB-Tg(ITGAM-DTR/EGFP)34Lan/J, Jax Stock # 006000) and C57BL/6J mice (Jax Stock # 00664).

2.2. Glucose sensors, implantation and murine continuous glucose sensor (CGM) system

All modified Navigator glucose sensors used in these *in vivo* studies were obtained from Abbott Diabetes Care (Alameda California). Glucose sensors were

* Corresponding author. Center for Molecular Tissue Engineering, University of Connecticut, School of Medicine, Farmington, CT 06030, USA. Tel.: +1 860 679 2859; fax: +1 860 679 2936.
E-mail address: klueh@nso.uchc.edu (U. Klueh).

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implanted into mice and continuous glucose monitoring (CGM) was undertaken as described recently [3–5]. Blood glucose reference measurements were obtained at least daily using blood obtained from the tail vein of the mouse and a FreeStyle® Blood Glucose Monitor. The Institutional Animal Care and Use Committee of the University of Connecticut Health Center (Farmington, CT) approved all the studies involving mice.

2.3. Glucose sensor function in macrophage deficient mice (op/op mice)

Heterozygous *op*/*op* breeding pairs were obtained from Jackson Lab, Bar Harbor, Maine. Homozygous MQ deficient and phenotypically normal littermates (heterozygous and homozygous) (LM) were derived from the breeding pairs. The *op*/*op* macrophage deficient mice are generally greater than 85% deficient in circulating monocytes [1,6]. Both the *op*/*op* macrophage deficient mice and normal littermates were evaluated in the murine CGM model described above. Normal littermates of the homozygous *op*/*op* mice served as controls for the *op*/*op* studies. Using these mice, the role of CSF-1 dependent M/MQ deficiency on sensor induced tissue reactions and sensor performance *in vivo* was determined.

2.4. Glucose sensor function in macrophage depleted human diphtheria toxin receptor (hDTR) chimeric mice

The development of transgenic mice expressing the diphtheria toxin receptor driven by a CD11b promoter provides an elegant method to selectively deplete MQ in mice [2,7–9]. Due to the CD11b driven expression of the human diphtheria toxin receptor on monocyte-macrophages, the addition of small intravenous dosages of diphtheria toxin to these transgenic mice triggers highly effective apoptotic destruction of all monocyte/macrophage populations for short-term studies. However for long-term macrophage depletion studies, chimeric DTR-CD11b mice are utilized to minimize toxicity and death of hDTR-CD11b mice due to long-term DT injections on resident macrophages [10]. The protocol for obtaining DTR-chimera mice involves injection of hDTR mouse derived bone marrow into irradiated normal LM mice (C57BL/6) (see Fig. 1). 4–8 weeks post bone marrow treatment this approach yielded a chimeric mouse with circulating DTR-monocyte/MQ (susceptible to DT) and resident tissue MQ, which are resistant to DT. Since only blood bone marrow derived monocyte/macrophages (M/MQ) are recruited to sites of sensor implantation this approach depleted circulating M/MQ systemically or locally by injection of DT. DT injection into the hDTR chimera mice generally reduces circulation monocytes to <50% of normal levels, but DT injections have no effects on normal mice. The resulting chimeric mice and control C57BL/6 mice were evaluated in the murine CGM model described above.

2.5. CGM data analysis for murine CGM models

Reference blood measurements and sensor output were used to calculate the mean absolute relative difference (MARD) over a four-week experiment for the three groups of mice *op*/*op*, hDTR and control mice [11]. Equations (1.1) through (1.3) describe the MARD calculation in detail. Sensitivity (S in mg/dL·nAmp) is calculated for each mouse experiment based on the reference blood glucose (BG) and the sensor output (I in nA) measurements in an initial reference stage of the experiment, i.e. k in Equation (1.2) is approximately 5, for the first initial 5 measurements across 2 days.

$$\mathrm{MARD(mean)} = \left(\left(\sum_i^n \frac{|\mathrm{CGM}_i - \mathrm{BG}_i|}{\mathrm{BG}_i}\right) \Big/ n\right) \times 100\% \tag{1.1}$$

$$\mathrm{Sensitivity}(S) = \left(\left(\sum_i^k (\mathrm{BG}_i / I_i)\right) \Big/ k\right) \tag{1.2}$$

$$\mathrm{CGM}_i = S \times I_i \tag{1.3}$$

Since most of the mean MARD values were non-normal in distribution, Kruskal–Wallis tests were used to conduct statistical comparisons among the 3 groups of MARD values, as a non-parametric equivalent to analysis of variance (ANOVA). Mann–Whitney U tests were conducted to determine the statistical differences between pairs of average mean MARD values, as non-parametric equivalents to student t-tests. Those variables that were normally distributed were the MARD values for mice that were normal heterozygous littermates of the homozygous *op*/*op* macrophage deficient mice (MQ-LM), Chimeric with No DT injection (for all time selections), and C57BL/6 DT injected (for all time selections except week 2).

2.6. Histopathologic analysis of tissue reactions at glucose sensor implantation sites

In order to evaluate tissue responses to glucose sensor implantation at various time points, individual mice were euthanized and the full thickness of the skin and sensors were removed enbloc in approximately 3 × 3 cm sections and immediately placed in tissue fixative. Tissue was fixed in zinc buffer for 24 h, followed by standard processing, embedded in paraffin, and sectioned. The resulting 4–6 μm sections were then stained using standard protocols for hematoxylin/eosin stain (H/E). Histopathologic evaluation of tissue reactions at sites of sensor implantation was performed on mouse specimens obtained from 1 to 28 days post-sensor implantation. The tissue samples were generally examined for signs of inflammation, including leukocyte influx, fibrosis, angiogenesis, and vessel regression. To provide an initial evaluation of the inflammatory reactions at the sensor tissue interface we utilized semi-quantitative evaluation scoring system from 0 to 4. For this system the tissue reactions were scored as follows:

0 = no inflammation (no leukocyte infiltration present near the implanted sensor),
1 = trace inflammation (occasional leukocyte infiltration present near the implanted sensor),
2 = mild inflammation (scattered and consistent leukocyte infiltration present near the implanted sensor),
3 = moderate inflammation (significant leukocyte infiltration near the implanted sensor),
4 = severe inflammation (dense leukocyte infiltration near the implanted sensor).

The individual histologic sections were evaluated in a double blind fashion and the mean inflammation index was determined. Since most of the average inflammation index values were non-normal in distribution, Kruskal–Wallis tests were

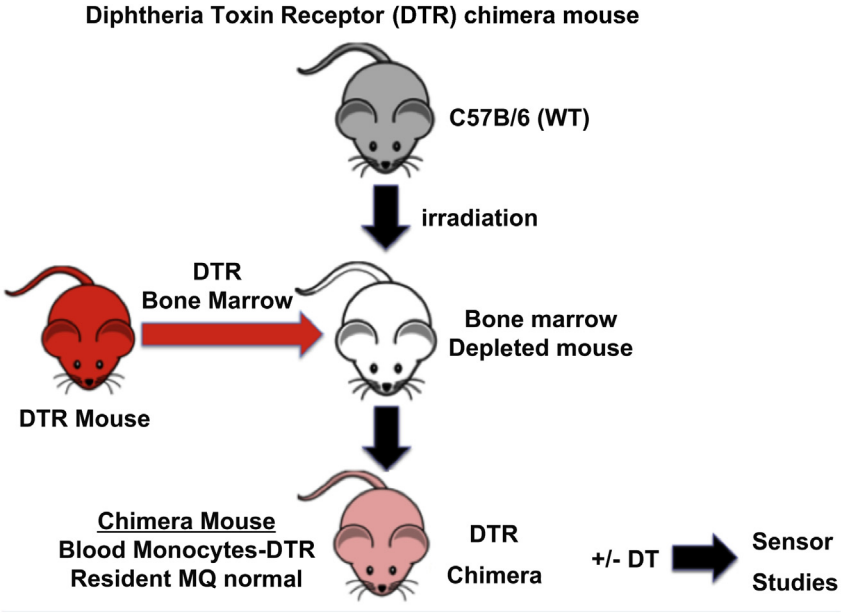

Fig. 1. Diagram of protocol used to obtain DTR-chimera mice. Fig. 1 represents the protocol for the formation of diphtheria toxin receptor chimeric mice used for the CGM studies presented in this application.