Concise Report

Mouse model of dermal fibrosis induced by one-time injection of bleomycin-poly(\(L\)-lactic acid) microspheres

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Objective. Animal models are useful tools to study various aspects of human diseases. Bleomycin (BLM)–induced scleroderma mouse has been widely investigated as an animal model of scleroderma. Repeated injections of BLM, either daily or every other day, for 3–4 weeks are required to induce scleroderma in mice. Poly(\(L\)-lactic acid) (PLA) is a biodegradable, biocompatible and bioabsorbable device that has been widely investigated for controlled drug release. In this study, we fabricated BLM-containing PLA microspheres and subcutaneously injected them into C3H mice for only one time.

Methods. Treated skins were harvested at days 7 and 21. Then, histological examination and collagen content measurement assay were performed. The mRNA expression of \(\alpha(1)\) collagen (COL1A1), monocyte chemoattractant protein-1 (MCP-1), TGF-\(\beta_1\) and connective tissue growth factor (CTGF) were quantified by real-time PCR.

Results. Dermal fibrosis was histologically observed at day 7 after injection and remained present at day 21. Tissue responses against BLM-PLA microspheres alone were mild. Soluble collagen content and expression level of \(\alpha(1)\) collagen mRNA were significantly elevated at day 7 and expression levels of MCP-1 mRNA and TGF-\(\beta_1\) mRNA at day 7 and CTGF mRNA at day 21 were also elevated.

Conclusion. The present study demonstrated for the first time that one-time injection of BLM-PLA microspheres can induce dermal fibrosis in C3H mice. BLM-PLA microspheres thus offer a labour-saving, simple and powerful tool to establish an animal model of BLM-induced dermal fibrosis.

Key words: Bleomycin, Scleroderma, Mouse model, Dermal fibrosis, Drug delivery system, Poly(\(L\)-lactic acid).

Introduction

SSc is a CTD characterized by extensive fibrosis associated with increased deposition of extracellular matrix (ECM) proteins in the skin and various internal organs, vascular injury and immunological abnormalities\([1, 2]\). To elucidate the pathogenesis and develop novel treatments, animal models are very valuable. As mouse models of systemic sclerosis, tight skin mouse, sclerodermatous graft-versus-host disease mouse and bleomycin (BLM)-induced skin fibrosis/scleroderma mouse have been extensively studied\([3, 4]\). Recent reports have described new murine models of scleroderma, involving injection of TGF-\(\beta\) and connective tissue growth factor (CTGF)\([5]\), a modified model of sclerodermatous graft-versus-host disease mouse\([6]\) and relaxin gene knockout mouse\([7]\).

Animal models of scleroderma induced by repeated subcutaneous injections of BLM have been established in various mouse strains\([8]\). These mice show dermal sclerosis comprising thickened and homogeneous collagen bundles, and pulmonary fibrosis. The present study established a simple and labour-saving method using poly(\(L\)-lactic acid) (PLA) microspheres containing BLM. This method requires a one-time injection of BLM, instead of repeated daily injections for 4 weeks.

Materials and methods

Preparation of BLM-PLA microspheres

PLA (molecular weight, 20000) was purchased from Wako Pure Chemical Industries (Osaka, Japan). BLM was kindly supplied by Nippon Kayaku (Tokyo, Japan). PLA microspheres containing BLM were prepared by the solvent evaporation method with water-in-oil-in-water (W/O)/W emulsion\([9]\). Briefly, 200 \(\mu\)g of BLM dissolved in water was poured into 5 ml of methylene chloride containing 200 mg of PLA microspheres, followed by emulsifying probe sonication to form a W/O emulsion. The emulsion was added to 10 ml of a 1 wt% polyvinyl alcohol (PVA; weight-average molecular weight 90 000; polymerization degree 1800; degree of saponification, 88 mol%) solution saturated with methylene chloride at room temperature and agitated using a vortex mixer to form a (W/O)/W double emulsion. The double emulsion was stirred using an impeller (200 r.p.m.) at room temperature until methylene chloride was completely evaporated. Microspheres were collected by centrifugation (3700 \(g\), 5 min, 4°C), washed three times with cold distilled water and finally lyophilized. For control, H\(_2\)O-PLA microspheres were prepared. To infer \(\text{in vivo}\) drug-release pattern, concentration of BLM incorporated in the microspheres and \(\text{in vitro}\) release from microspheres were measured with high-performance liquid chromatography as described\([9]\). All samples were run in triplicate.

BLM-PLA microsphere treatment

For the next step, 0.5 mg of BLM-PLA microspheres containing 0.013 mg of BLM was suspended in 400 \(\mu\)l of PBS. Microspheres in phosphate-buffered saline (PBS) (400 \(\mu\)l) were subcutaneously injected into shaved back skin at one site of 8-week-old female C3H mice. Control mice were injected with control-PLA microspheres.

Histological examination

Skin samples were fixed in 10% formalin solution and embedded in paraffin. Haematoxylin–eosin staining was performed on each section.

Collagen assay

Soluble collagen content of skin was determined using a Sircol collagen assay kit (Biocolor, Belfast, UK) according to the instructions of the manufacturer.
**Real-time PCR**

The mRNA expression of αI(I) collagen (COL1A1), MCP-1, TGF-β1 and CTGF was quantified using a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), using the Taq Man Universal PCR Master Mix and pre-designed Taq Man Gene Expression Assay primers/probe set for mouse COL1A1: Mm00801666_g1, MCP-1: Mm00441242_m1, TGF-β1: Mm00441724_m1 and CTGF: Mm00515790_g1 (Applied Biosystems). All results were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Ethics approval was obtained by the Animal Care Committee of Gunma University for all animal experiments.

**Statistical analysis**

All data are expressed as mean ± S.D. Significance testing was assessed using the Mann–Whitney U-test. Values of \( P < 0.05 \) were considered statistically significant.

**Results**

**Size of microspheres, amount of BLM in microspheres and in vitro drug release**

Microspheres were 20–40 μm in diameter. The amount of BLM in 1 mg of microspheres was 26.1 ± 0.8 μg. The percentages of BLM release in vitro from PLA microspheres were 58 ± 6.9, 66 ± 0.6, 72 ± 0.0, 78 ± 0.0 and 83 ± 0.0% within 4, 7, 14, 21 and 28 days, respectively.

**Histological examination**

By day 7, the skin of BLM-PLA microsphere-injected mice (BLM-m mice) was thickened with increased collagen bundles in both dermal and adipose tissue (Fig. 1b). Abundant inflammatory cells were observed in the adipose tissue of BLM-m mice (Fig. 1c). In control-PLA microsphere (without containing BLM)-injected mice (control-m mice), cell infiltration was also observed, but dermal thickening was not induced (Fig. 1a). By day 21, both dermal fibrosis and increased dermal thickness were obvious in the skin of BLM-m mice (Fig. 1e), as compared with control-m mice skin (Fig. 1d). Collagen bundles appeared to be markedly thickened with partial replacement of subcutaneous adipose tissue. Some microspheres were identified as small round particles, around which mononuclear cells had infiltrated (Fig. 1f). In control-m mice, several microspheres were also seen with mononuclear cell infiltration (Fig. 1d). Dermal thickness in BLM-m mice displayed an increase of about 1.4-fold as compared with control-m mice at day 7 (239 ± 33 μm vs 167 ± 20 μm; \( P < 0.05 \)) and a 1.9-fold increase at day 21 (295 ± 64 μm vs 153 ± 10 μm; \( P < 0.05 \)).

**Acid-soluble collagen content**

In lesional skin of BLM-m mice, soluble collagen content (144 ± 12 μg/5 mm punch-biopsied skin) was increased as compared with control-m mice (105 ± 18 μg) at day 21 (\( P < 0.05 \)) (Fig. 2a).

**mRNA expression of COL1A1**

Real-time PCR analysis showed a 2.2-fold elevation of COL1A1 mRNA expression in the skin of BLM-m mice as compared with control-m mice at day 7 (1.01 ± 0.38 vs 0.46 ± 0.13; \( P < 0.05 \)) and a 3.1-fold elevation at day 21 (1.06 ± 0.40 vs 0.34 ± 0.18; \( P < 0.05 \)) (Fig. 2b).

**mRNA expression of MCP-1, TGF-β1 and CTGF**

The expression level of MCP-1 mRNA was 10-fold increased in the skin of BLM-m mice as compared with control-m mice skin were 53.16 ± 27.10 μg/5 mm punch-biopsied skin) was increased as compared with control-m mice at day 7 (1.01 ± 0.38 vs 0.46 ± 0.13; \( P < 0.05 \)) at day 21, the expression levels were markedly decreased in control-m mice skin (2.43 ± 1.34) and BLM-m mice skin (2.76 ± 1.62) (Fig. 2c).

The expression level of TGF-β1 mRNA in the skin of BLM-m mice was 1.3-fold higher than that of control-m mice (6.06 ± 0.39 vs 4.47 ± 0.80; \( P < 0.05 \)). These levels showed 2.8-fold and 3.8-fold elevation as compared with untreated mice (1.58 ± 0.28) in the skin of control-m and BLM-m mice, respectively. At day 21, TGF-β1 levels in both control and BLM-m mice skin were decreased to the level comparable with untreated mice (Fig. 2d).

There were not apparent differences among the expression level of CTGF mRNA in the skin of untreated, control-m and BLM-m mice at day 7 (33 ± 8 μg/5 mm punch-biopsied skin) was increased as compared with control-m mice (2.04 ± 0.42 vs 0.95 ± 0.33; \( P < 0.05 \)) (Fig. 2e).

**Discussion**

Yamamoto and others [8, 10] established a mouse model of scleroderma by repeated subcutaneous injections of BLM. The induction of dermal sclerosis by BLM is considered to be, in part, mediated by inflammatory and fibrogenic cytokines, in addition to the direct

![Fig. 1. Histopathological evaluation. C3H mice were injected with control-PLA microspheres (a, d) and with BLM-PLA microspheres (b, c, e, f). The lesional skin at 7 days after injection (a–c) and at 21 days after injection (d–f). Bars in the left and middle panels, 200 μm; right panels, 100 μm. Microspheres are indicated by arrows. Each treatment group includes at least four mice.](https://academic.oup.com/rheumatology/article-abstract/47/4/454/1791640/455)
effect of BLM on ECM synthesis in fibroblasts [11]. In their model, they injected 100 μl of BLM solution (0.1–1 mg/ml) at the same site daily or every other day for 3–4 weeks. Total amount of BLM was estimated to reach 150–2800 μg. At first, we tried their method to use the BLM-induced scleroderma for another purpose. However, as fibrosis gradually developed, it became more difficult to inject BLM solution at the same site and we could not get a good result. Therefore, we modified their method and performed a one-time injection of BLM-PLA microspheres containing only 13 μg of BLM. In our study, a single injection was able to induce dermal fibrosis in C3H mice, a BLM-susceptible strain [12].

For the purpose of controlled release of the drug, PLA, poly(glycolic acid) (PGA) and poly(lactide-co-glycolide) have been extensively investigated as drug carriers [9, 13–15]. PLA and PGA are biodegradable, biocompatible and bioabsorbable. The degraded products are non-toxic, non-carcinogenic and non-teratogenic [16]. Upon hydrolysis, PLA yields lactic acid, and this acid then enters the tricarboxylic acid cycle and is excreted from the body as water and carbon dioxide [17]. In the clinical setting, these compounds are widely used as absorbable surgical sutures, orthopaedic applications and drug carriers [18]. We thus applied the concept of drug delivery system to our experiments. Since BLM solution might have rapidly diffused from the injection site in previous experiments, repeated injections were required. In contrast, BLM-PLA microspheres enabled BLM to be efficiently and continuously released at the injection site, thus reducing both the number of injections and the amount of BLM to be injected.

Histological examinations confirmed that dermal thickening and fibrosis had been induced at day 7 after BLM-PLA microsphere injection, and these findings became more prominent by day 21 (Fig. 1). Yamamoto et al. [8] reported that dermal sclerosis was sustained for at least 6 weeks after cessation of treatment [8]. In the present study, dermal thickening was still observed at 6 weeks, although the thickness was decreased as compared with that at day 21 (data not shown).
Even if PLA is biocompatible, tissue responses are elicited against the microspheres [19]. In our study, numerous microspheres surrounded by inflammatory cells were observed in both control-m and BLM-m mice skin at day 7. At day 21, a small number of microspheres with slight mononuclear cell infiltration remained in the lower dermis and adipose tissue of BLM-m and control-m mice skin (Fig. 1). Tissue responses against microspheres themselves were so mild that the effect of microspheres on inflammation and fibrosis could be ignored.

The amount of newly synthesized collagen and expression of COL1A1 mRNA were elevated even at day 21 (Fig. 2a and b), when BLM-release was estimated to be very low. These data suggest that once the process of fibrosis starts, it would be kept with low concentration of BLM.

MCP-1 is known to be an important chemoattractant for mononuclear cells during inflammation [20], and has been shown to be up-regulated in various fibrotic conditions [21–25]. In our model, MCP-1 expression in BLM-m mice skin was significantly elevated at day 7. At day 21, the levels in both control-m and BLM-m mice skin had returned to the level of untreated skin (Fig. 2c).

TGF-β plays a key role in the pathogenesis of scleroderma, acting as a strong chemoattractant for fibroblasts and stimulating the production of ECM proteins such as collagens and proteinase inhibitors [26]. In BLM-induced fibrotic skin, TGF-β was immunohistochemically detected in infiltrating cells and fibroblasts [8], and both the expression and synthesis of TGF-β1 were enhanced [27]. We demonstrated the elevation of TGF-β1 in both BLM-m and control-m mice skin at day 7 (Fig. 2d).

CTGF has recently received much attention as a possible key determinant of progressive fibrosis and excessive scarring. CTGF increases fibroblast proliferation and ECM synthesis, and is a downstream mediator of TGF-β1 and plays a key role in mediating ECM production by fibroblasts 

References