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Differentiated tonsil-derived mesenchymal stem cells embedded in Matrigel restore parathyroid cell functions in rats with parathyroidectomy

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ABSTRACT

Parathyroid cells release parathyroid hormone (PTH), which controls calcium homeostasis. Loss of parathyroid cells results in hypoparathyroidism and consequent low-turnover bone disease. Here, we investigated whether our recently-established human tonsil-derived mesenchymal stem cells (TMSC) restore *in vivo* parathyroid cell function in rats with parathyroidectomy (PTX). Compared with undifferentiated control TMSC, TMSC differentiated with activin A and soluble sonic hedgehog induced a significant release of PTH as early as day 7, with increased PTH release occurring in response to lower calcium levels and *vice versa*. Released PTH increased osteocalcin expression and alizarin red S staining in preosteoblastic cells, indicating its functional activity. PTX rats fed calcium-free diet only survived for ~10 days. Subcutaneous injection with TMSC alone did not increase their survival rates, regardless of differentiation. However, survival rates increased for up to 28 days in response to TMSC embedded in Matrigel (TMSC-MA), showing 40% and 80% in control and differentiated TMSC-MA, respectively. When compared with continuous increases by control TMSC-MA, stable levels of secreted PTH and serum ionized calcium were found in PTX rats with differentiated TMSC-MA. This is the first report that differentiated TMSC resemble parathyroid cells and, if embedded in Matrigel, restore *in vivo* parathyroid function.

1. Introduction

The increasing incidence of thyroid cancer has become epidemic in the United States and other developed countries, including Korea [1]. Thyroidectomy is not unusual, and its concomitant surgical parathyroid loss is likely to be inevitable. Regardless of whether it is therapeutic or unintentional, loss of the parathyroid results in hypoparathyroidism and consequent low-turnover bone disease showing chronic hypocalcemia [1].

Hypoparathyroidism is an uncommon endocrine-deficiency characterized by low serum calcium levels, elevated serum phosphorus levels, and the absence of circulating parathyroid hormone (PTH) [2,3]. PTH, parathormone or parathyrin is secreted from the parathyroid (chief) cells of the parathyroid glands as a polypeptide that contains 84 amino acids and acts as the major hormone regulating calcium homeostasis in the body [4]. The minute-to-minute regulation of serum ionized calcium (iCa^{+2}) is exclusively regulated through PTH. In turn, PTH secretion is also regulated by

Abbreviation: CaSR, calcium sensing receptor; CHGA, chromogranin; CM, conditioned medium; cCM, CM collected from the non-differentiated control TMSC; dCM, CM collected from the differentiated TMSC; iCa, ionized calcium; MA, Matrigel; O-dM, osteogenic differentiation medium; PTH, parathyroid hormone; PTX, parathyroidectomy; TMSC, tonsil derived mesenchymal stem cells; cTMSC, non-differentiated control TMSC; dTMSC, differentiated TMSC.

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serum calcium levels through specific calcium-sensing receptor (CaSR) on the surface of parathyroid cells [5].

Teriparatide (Forteo; Eli Lilly & Co., Indianapolis, IN), a 1-34 N-terminal fragment of PTH peptide (PTH(1-34)), was recently developed for the purpose of PTH replacement, and it is currently commercially-available for treatment of osteoporosis [6]. Teriparatide has also been tested to determine whether this drug can be applied to hypoparathyroidism treatment. For example, short-term controlled trials for 10–14 weeks with once or twice-daily injection of PTH(1-34) safely and effectively managed hypoparathyroidism and hypocalcemia [7,8]. Furthermore, long-term treatment for 3 years with twice-daily subcutaneous injection of PTH(1-34) was found to maintain normal serum calcium concentrations safely and effectively. In addition to PTH(1-34), treatment for 24 months with subcutaneous injection of the full sequence of PTH peptide, PTH(1-84), also led to significant decreases in calcium and 1,25-dihydroxyvitamin D requirement in patients with hypoparathyroidism without altering serum and urinary calcium levels [9]. Although recent clinical pilot studies showed evidence of the therapeutic efficacy of PTH(1-34) and PTH(1-84), their uses still require further investigation to overcome several limitations, such as the short half-life (~4 min) of PTH [4] and complications including impaired renal function after PTH treatment for 10 weeks [7,8]. Accordingly, better therapeutic strategies should be re-established to achieve physiological levels of PTH for a long time and minimize their potential adverse effects, particularly against bone. As a result, studies to enable the manufacture of either long-acting PTH or methods enabling its sustained release have recently been suggested.

In addition to direct PTH replacement, autografting of parathyroid tissues from patients has been used to treat hypoparathyroidism. In this method, damaged or isolated parathyroid tissues can be mechanically minced into small pieces, inserted into small pockets, and then implanted into intramuscular areas. However, this autografting is only available when the damaged parathyroid is recognized [10,11]. Furthermore, it is known that grafted parathyroid tissue is likely to exhibit normal function 6–10 weeks post-implantation, but it is still unknown whether or how long graft tissue survives in the body. Moreover, most parathyroid tissue to be autografted is obtained from patients with thyroid cancer who have already received I^{134} radiation therapy; therefore, parathyroid tissue is likely to have radiation-induced gene mutations. Accordingly, it is difficult to ensure the normal functionality of implanted parathyroid tissue after its successful implantation. Nonetheless, autografting of the discarded parathyroid tissues together with application of multiple high doses of combined vitamin D (1.25–5 mg/d) and calcium (1–9 g/d every 6 h) is currently considered the best available therapy [11]. It should also be noted that patients with hypoparathyroidism who are treated with a combination of calcium and vitamin D for a considerably long time undergo irritated hypercalciuria, leading to renal problems and vitamin toxicity. Owing to these issues, another therapy such as a direct replacement of the functional parathyroid without taking multiple high doses of vitamin D and calcium is anticipated.

Stem cells have recently received increased attention owing to their potential for use in tissue engineering and clinical applications [12,13]. Stem cells are defined as self-renewable, multipotent progenitor cells with the capacity to differentiate into several distinct lineages [14]. It was previously reported that human embryonic stem cells (hESC) [15,16] and differentiated thymic stromal cells [10] could be used for the *in vitro* regeneration of parathyroid-like cells. However, those cells have critical limitations for a clinical use. Specifically, there are ethical issues associated with the use of hESC, while isolation of thymic stromal cells is rather invasive, and it takes over 10 weeks for thymic stromal cells to differentiate and secrete PTH. We previously isolated new stem cells from palatine tonsillar tissues, tonsil-derived mesenchymal stem cells (TMSC), and reported that TMSC have the potential to differentiate into endoderm tissues expressing the PTH gene [17]. In this study, we further investigated whether TMSC can be used for the development of functional parathyroid cells *in vitro* and *in vivo*. Our results showed for the first time that, after differentiation for significantly shorter times (7–10 days), TMSC release PTH, which is responsive to extracellular calcium levels. Furthermore, released PTH is functional towards osteogenicity. Finally, when cultured in three dimensions with Matrigel, differentiated TMSC showed almost complete recovery of survival rates and restored serum levels of PTH and iCa^{2+} in our animal model (i.e. parathyroidectomized (PTX) rats fed calcium-free diet).

2. Materials and methods

2.1. Isolation and culture of TMSC

TMSC isolation and culture were conducted as previously described [17]. Briefly, a total of five patients younger than 10 years undergoing tonsillectomy (3 boys and 2 girls, mean age 6.2 years) were recruited at random from consecutive patients presenting between April 2012 and December 2012 at the Department of Otorhinolaryngology–Head and Neck Surgery, Ewha Womans University Medical Center (EWUMC, Seoul, Korea). Informed written consent was obtained from legal guardians of all patients participating in this study, and the study protocol was approved by the EWUMC institutional review board. Following tonsillectomy, the tonsillar tissue was surgically removed, chopped and digested in RPMI-1640 (Invitrogen Corp., Carlsbad, CA) containing 210 U/mL collagenase type I (Invitrogen) and 10 μg/mL DNase (Sigma–Aldrich, St Louis, MO) for 30 min at 37 °C. Digested tissue was subjected to filtration through a wire mesh, after which the cells were washed twice in RPMI-1640/20% normal human serum (NHS; PAA Laboratories GmbH, Pasching, Austria) and once more in RPMI-1640/10% NHS. Adherent mononuclear cells were obtained by Ficoll–Paque (GE Healthcare, Little Chalfont, UK) density gradient centrifugation. Cells were then plated at a density of 1×10^8 cells in a T-150 culture flask in DMEM containing high (4500 mg/L) glucose (DMEM-HG) (Welgene Inc., Korea), 10% FBS (Invitrogen), 100 μg/mL streptomycin and 100 U/mL penicillin. After 48 h, non-adherent cells were removed from the medium and adherent mononuclear cells (hereafter called TMSC) were replenished with new culture medium. All TMSC used in this experiment were passage 3–9.

2.2. Differentiation into parathyroid-like cells

TMSC were differentiated into parathyroid-like cells using the modified Bingham protocol [15], as previously described [17]. Briefly, cells were incubated in DMEM with 10% FBS until they reached 90% confluency, at which time they were treated with differentiation medium containing activin A (100 ng/mL) and soluble sonic hedgehog (Shh, 100 ng/mL) for 7–21 days. Differentiation medium also included normal levels of $CaCl_2$ (1.8 mM) unless otherwise stated, and was changed every 3–4 days. Following differentiation, cells and their conditioned medium (CM) were analyzed to determine the level of PTH protein expression and the secreted PTH protein, respectively.

2.3. Measurement of secreted PTH protein concentration

CM was collected from each time point during the differentiation period, filtered using a 0.2 μm syringe filter, and stored at −80 °C until use. Each CM collected from the non-differentiated