Analysis of physiological activity of carboxymethyl-chitin

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Section 0.

First introduction

In the present study, we investigated the physiological activity of carboxymethyl-chitin, CM-chitin. Physiological activities of chitin have been reported in many previous researches, however, there is still controversy over the functions and we have plenty of scope for investigating. At this point, we performed three separate experiments of CM-chitin. Firstly, we examined the stimulation activity of CM-chitin in peritoneal cavity of mouse. Secondly, we examined whether CM-chitin promotes *in vitro* chondrogenesis. Finally, we examined bactericidal function of CM-chitin. First, second and third experiments were allocated into Section 1, 2, 3, respectively. Each section consisted of seven subsections: Abstract, Introduction, Materials and Methods, Results, Discussion, Figures and figure captions, References. Conclusion of this study was allocated in the end of this dissertation.

Section 1.

Biological roles of carboxymethyl-chitin associated for the growth factor production

1-1. Abstract

Many techniques to restore cartilage defection have been tried to. However, the development is still under way because of problems, including loosening of artificial joint, degenerative change of compensated tissue, risk of viral transmission via allograft/autograft, and cost of therapeutic materials for repair. In the previous research, we found that complementing cartilage defective part with carboxymethyl-chitin (CM-chitin)/β-tricalcium phosphate (β-TCP) composite induced regeneration of cartilage in rabbits *in vivo*, and it is presumable that CM-chitin plays a key role in chondrogenesis causing the regeneration of cartilage. However, the induction mechanism of chondrogenesis with CM-chitin is still unclear. In this study, we investigated the cell responses to CM-chitin by using peritoneal exudate cell (PECs) in mice and found that CM-chitin induced the expression of inflammatory cytokines and growth factors, both of which are both considered to correlate with chondrogenesis. After intraperitoneal injection of CM-chitin showed enhanced expressions of mRNA of interleukin-1β (IL-1β), interleukin-6 (IL-6), keratinocyte-derived chemokine (KC), tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1), in PECs were observed by reverse transcriptase polymerase chain reaction (RT-PCR). Productions of TGF-β1 protein were confirmed by enzyme linked immunosorbant assay (ELISA). It was also shown that mononuclear cells in PECs were responsible for the TGF-β1 production. These results suggest that CM-chitin is an inductor of inflammatory cytokines and growth factors and may contribute to regeneration of cartilage.

1-2. Introduction

In the human organism, cartilage has poor capacity to repair because of its low mitotic activity and avascular nature, and it is difficult to regenerate normal cartilage at injured joint [1,2]. Cartilage is mainly composed of hyaline cartilage, which involves a rich extracellular matrix and water [3]. However, osteochondral defects that penetrate the subchondral bone usually heal with tissue that contains fibrous cartilage which lacks the durability and many of the mechanical properties of hyaline cartilage [4]. Several methods have been carried out for medical treatment of cartilage; the substitution with artificial joint, the bone marrow permeation by subchondral drilling, and abrasion arthroplasty were used for the treatments. However, these methods still have some problems, such as the loosening of artificial joint, the regeneration of fibrous cartilage from bone marrow spinal fluid, and the degenerative change of compensated tissue [5].

In this context, tissue-engineering techniques have been developed for regeneration of cartilage at the affected part. Tissue-derived biomaterials such as cultured-autograft or allograft were used for repairs of cartilage defect: transplantation of the tissue showed well regeneration of cartilage tissue [12]. However, many problems still remain including a risk of viral transmission via allograft or a lack of tissue for transplantation, which can hardly be harvested from patient body [6,13]. Biomaterials as scaffolds constituting the organization structure and growth factors promoting cell differentiation or proliferation are also used for induction of regeneration at defective cartilage [6-8]. The scaffold materials such as hydroxyapatite [9,10] have been used to achieve repair small osteochondral defects. However, this may not be a widely acceptable method for complete repair of hyaline cartilage [11]. Additionally, the cartilage repairing material involving the scaffolds and the growth factors associated with chondrogenesis is too expensive for practical treatment of all patients.

In the previous study, we found that complementing the osteochondral defecting part with the carboxymethyl-chitin (CM-chitin)/β-tricalcium phosphate (β-TCP) composite induced the regeneration of cartilage *in vivo* of rabbits (Figure1-1) [14]. In the investigation, the regeneration of articular cartilage was confirmed with CM-chitin/β-TCP but not with β-TCP alone at eight weeks after implantation,

presuming that CM-chitin played a key role for the regeneration of cartilage. However, the induction mechanism of chondrogenesis with CM-chitin was unclear. Chondrocyte is known to be differentiated from bone marrow-derived mesenchymal stem cells, by the effect of growth factors, such as transforming growth factor (TGF) or bone morphogenetic protein (BMP) [6,8,15]. Since CM-chitin contains no growth factor, this material may stimulate surrounding cells at the region of administration to induce those factors. Further, it is considered that fracture-healing process is associated with the initial inflammation caused by inflammation cytokines and with the following chondrogenesis and osteogenesis [16,17]. In the present study, we investigated the expression of inflammatory cytokines and growth factors induced by the peritoneal injection of CM-chitin in mice.

1-3. Materials and Methods

1-3-1. *Stimuli and cells*

The CM-chitin (degree of substitution for O-carboxymethylation = 79 mol%, degree of deacethylation = 27 mol%) was prepared from chitin extracted from Queen Crab shells according to the method reported previously [18]. Soluble-type and gel-type CM-chitins were prepared as described [14], and β-TCP granules were purchased from Taihei Chemicals (Saitama, Japan). The levels of endotoxin contamination were determined by Endospecy® test according to the manufacture's instruction.

Soluble-type CM-chitin was dissolved in PBS (10 mg/ml) and gel-type CM-chitin and β-TCP granules were suspended in PBS (10 mg/ml). Six to 8-week-old male BALB/cN sea mice were obtained from Kyudo (Saga, Japan). The animals received humane care in accordance with our institutional guidelines and the legal requirements of Japan. Mice were injected intraperitonealy with 0.5 ml of stimulus solution or suspension. Peritoneal exudate cells (PECs) were collected from respective mice in each stimulation time, at 1, 2, 4, 24 or 72 h after injection. The numbers of cells collected were 1-3 × 10^6 cells/mouse. The PECs from mice were subjected to the extraction of ribonucleic acid (RNA) or cultured for the detection of secreted proteins.

1-3-2. *mRNA expression*

Total cellular RNA was extracted from PECs of 2 mice for each treatment, using TRIzol® Reagent (Invitrogen Inc., Carlsbad, CA, USA) or GenElute™ Mammalian Total RNA Kit (Sigma-Aldrich Corp., St. Louis, MO, USA) according to the manufacturer's procedure. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with total RNA (≦500 ng) using RNA PCR Kit (AMV) Ver.3.0 (TaKaRa BIO Inc., Shiga, Japan) with sense and antisence oligonucleotide primers specific for β-actin [19], interleukin-1β (IL-1β) [20], IL-6 [20], keratinocyte-derived chemokine (KC) [21], tumor necrosis factor-α (TNF-α) [20], macrophage-colony stimulating factor (M-CSF) [22], receptor activator of NF-κ B (RANKL) [23], interferon-γ (IFN-γ) [20], inducible NO synthase (iNOS) [24], cyclooxygenase-2 (COX-2) [25] and TGF-β1 [26]. The amount of RNA was corrected by β-actin expression. The amplified products were detected by electrophorsis on a 1%

agarose gel.

1-3.3. *Growth factor assay*

PECs were fractionated by the density gradient method using Histopaque-1083/1119 (Sigma-Aldrich) to obtain mononuclear cells (MNC) fraction. The numbers of cells obtained were 3-7 × 10^5 /mouse. Total PECs collected from one mouse or MNC from two mice were suspended in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 200 μg/ml of BSA (Nacalai Tesque Inc., Kyoto, Japan) and plated on each well of 6-well plate. The cells were incubated for 24 h at 37°C in humidified air containing 5% CO_2. After incubation, culture supernatants were collected and analyzed for secreted TGF-β1 (R&D Systems Inc., Minneapolis, MN, USA) using an enzyme linked immunosorbant assay (ELISA) kit according to the manufacturer's instruction. Data are the mean ± SE of three independent experiments. Serum TGF-β1 concentrations in different experimental groups were analyzed for statistical significance by using Welch's t-test.

1-4. Results

1-4-1. *Stimulation of murine PECs by soluble-type CM-chitin*

It was hypothesized in our previous experiment [14] that CM-chitin gel in the CM-chitin/β-TCP composite stimulated cells located at the surroundings of the implanting lesion to produce cytokines and/or growth factors, and lead to promote the regeneration of cartilage. Since CM-chitin gel is a biodegradable material, which can be digested by lysozyme *in vivo* [27,28], the resulting soluble CM-chitin fragments may act as a stimulant for the regeneration. However, it is unknown whtat kind of cells are involved in the stimulation by CM-chitin. Thus, we first investigated the stimulating effect of soluble-type CM-chitin on cells *in vivo*. Soluble CM-chitin was intraperitoneally injected to BALB/c mice and mRNA expression in the stimulated PECs was detected by RT-PCR. Expression of mRNA for inflammatory cytokines, IL-1β, IL-6, KC, TNF-α, iNOS and COX-2 in PECs stimulated with CM-chitin were significantly enhanced in comparison with those with PBS as a negative control (Figure 1-2 a-i). Slight expression of these cytokines were observed in PBS injected mice at 1 h probably due to the injury of needle stick. In contrast, sustained inflammation was observed up to 24 h in CM-chitin injected mice PECs. The levels of endotoxin contamination was under the detection limit (less than 100 pg/mg). These suggested that CM-chitin was responsible for the expression of inflammation cytokines in mice.

Expression patterns of mRNA of TGF-β1 were also significantly different between two groups (Figure 1-2 j). The expression of TGF-β1 in PBS group kept in low level throughout the period was observed, while that in CM-chitin group was enhanced. The production of TGF-β1 protein was confirmed by ELISA. The PECs harvested at 1 to 72 h after injection of stimuli were cultured for the secretion of protein in serum free medium and the secreted TGF-β1 was analyzed (Figure 1-3). The result showed that TGF-β1 concentration from PECs stimulated with CM-chitin was significantly higher than that with PBS ($p<0.01$ for 24 h and $p<0.05$ for 1,4,48 h). TGF-β1 level at 24 h was clearly higher than that of 1 h ($p<0.05$), suggesting that TGF-β1 was secreted after inflammation (see Figure 1-2 a-d, h-i).

1-4.2. *Stimulation of murine PECs by gel-type CM-chitin*

In practical medical treatments, a gel-type CM-chitin/β-TCP composite material is used for the repairing of articular cartilage. Thus, we next investigated the effect of gel-type CM-chitin on PECs in mice. Expression of mRNA for inflammatory cytokines was enhanced up to 24 h after stimulation of gel-type CM-chitin in a similar manner as soluble-type CM-chitin (Figure 1-4 a-d). TGF-β1 was also induced by CM-chitin gel (Figure 1-4 e).

β-TCP was reported to be a stimulant for cytokine induction [29]. Expression of mRNAs of inflammatory cytokines by the stimulation of β-TCP granules was observed in agreement with the report. However, the expression level by β-TCP was lower than that by CM-chitin, especially for IL-6, KC, TNF-α, and TGF-β1 (Figure 1-4). The levels of TGF-β1 protein secretion was determined (Figure 1-5). Gel-type CM-chitin induced high concentration of TGF-β1, while the level induced by β-TCP was similar to that by PBS. Further, TGF-β1 production stimulated with CM-chitin/β-TCP composite is slightly lower than CM-chitin only. These results suggested that CM-chitin but not β-TCP is a stimulant for TGF-β1.

1-4-3. *Contribution of mononuclear cells in cytokine induction by CM-chitin*

It is known that macrophages are critical in the successful wound healing by participating in inflammatory and following processes [30]. Regulatory role of macrophages in bone wound healing was also reported [31]. In both cases, MNC contributed to the secretion of growth factors. Therefore we investigated the contribution of MNC in TGF-β1 secretion. The PECs from soluble-type CM-chitin injected mice were subjected to a density gradient separation using Histopaque to fractionate mononuclear cell (MNC). As shown in Figure 1-6, TGF-β1 was secreted from the MNC fractionated from PECs at 24 and 48 h after injection of soluble-type CM-chitin.

1-5. Discussion

In this study we found that CM-chitin stimulated cells to induce inflammatory cytokines, IL-1β, IL-6, KC, TNF-α, iNOS, and COX-2, in mice. Since CM-chitin contained no detectable endotoxin, CM-chitin is considered to be responsible for the inflammation. IL-1β and KC expression was continued to around 24 h, probably due to the paracrine interaction of early inflammatory genes [32,33].

Some of these cytokines are considered to act as attractants of cells. KC is a mouse homologue of human chemokine CXCL1 [34]. Chemokines are known as potent attractants for leukocytes such as neutrophils and monocytes [35]. Since macrophages are shown to be essential for wound and bone healing, KC might be associated with cartilage regeneration. TNF-α is reported to facilitate bone repair process by stimulating the recruitment of mesenchymal stem cells [17]. Since mesenchymal stem cells can proliferate to chondrocytes, TNF-α may also participate in the repair of cartilage [17].

We also observed the production of TGF-β1 in PECs induced by CM-chitin. The functions of TGF-β, which primarily controls cell growth and differentiation for chondrocyte [36], were investigated previously. It has a protective effect on the extracellular matrix of human articular chondrocyte by stimulating tissue inhibitor of metalloproteinases-1 (TIMP-1) expression [37], or by decreasing procollagenase expression [38]. TGF-β1 is also reported to increase the production of the chondroitin/dermatan sulfate proteoglycan, promoting the formation of the extracellular matrix, which is required for chondrogenesis [39], and to promote the expression of several integrins, which mediate cell adhesion to other cells or to the extracellular matrix [40-42]. In the culture of mesenchymal stem cells, TGF-β1 was used to promote the chondrogenesis *in vitro* in combination with BMP-2 and/or dexamethasone [8,43-46]. Those results indicate that TGF-β1 secreted in mice with CM-chitin stimulation is a possible cause of chondrogenesis in the lesion. Interestingly, the mRNA expression and protein production of TGF-β1 were not observed by the stimulation of β-TCP stimulation. Masuda et al. reported that CM-chitin/β-TCP composite induced cartilage regeneration, but β-TCP did not [14]. These differences in cartilage repair may be associated with the cytokine expression profiles of CM-chitin and β-TCP. Further, addition of β-TCP did not promote TGF-β production by CM-chitin.

These results suggested the importance of CM-chitin in cartilage regeneration.

The cytokine expression profile of CM-chitin, transient inflammation up to 24 h, followed by TGF-β1 expression (Figs. 1-2,3,4), is similar to those of wound and fracture healing [16]. In addition to that, the result shown in Figure4 suggests that MNC were responsible for the TGF-β1 production in whole PECs. This suggestion also coincides with the previous report that TGF-β1 was secreted by activated MNC within an inflammatory tissue [47] Thus, the mechanisms of cartilage regeneration may be expected as follows. Cells such as MNC or mesenchymal stem cells are recruited from surrounding tissues, including bone marrow and vessel, in the inflammation period, although responsible cells for inflammation evoked by CM-chitin have been clarified. The migrated MNC concluded the inflammation by producing suppression cytokines, such as TGF-β1, and then the mesenchymal stem cells proliferated to chondrocytes to regenerate cartilage tissue. Many biodegradable materials have been used for the regeneration of articular cartilage, generally with the aid of growth factor proteins [15,48-51]. The CM-chitin based β-TCP composite was demonstrated to repair articular cartilage without addition of growth factor [14]. Here, we showed that CM-chitin stimulated the induction of growth factors *in situ*. This may indicate that the composite has a dual role as a scaffold and an inducer of growth factor.

1-6. Figures and figure captions

Figure 1-1. Time-dependent expressions of mRNA for cytokines in PECs treated with soluble-type CM-chitin. Results with soluble-type CM-chitin were compared with those with PBS. The numbers above the figures indicate elapsed times (hour) after injection of PBS or soluble-type CM-chitin in PECs in mice. IL-1β (a), IL-6 (b), KC (c), TNF-α (d), M-CSF (e), RANKL (f), IFN-γ (g), iNOS (h), COX-2 (i) and TGF-β1 (j) are surveyed by RT-PCR.

Figure 1-2. Effect of soluble-type CM-chitin on expression of TGF-β1 in PECs. Total PECs were harvested from mice at 1, 4, 24, 48 or 72 h after administration of CM-chitin or PBS cultured for 24 h, and their supernatants were used for ELISA to evaluate the expression of TGF-β1. Each value is the mean ± SE of 3 samples. * and ** indicate the significances at $p<0.05$ and $p<0.01$, respectively, compared with PBS treated group. ## indicates the significance at $p<0.01$, compared with soluble-type CM-chitin treated group at 1 h.

Figure 1-3. Time-dependent expressions of mRNA for cytokines in PECs treated with soluble-type CM-chitin. Expressions for solid gel type CM-chitin were compared with those for β-TCP, which constitute the biomaterial composite with CM-chitin. The numbers at the head of figure indicate elapsed times (hour) after injection of β-TCP or gel-type CM-chitin in PECs in mice. IL-1β (a), IL-6 (b), KC (c), TNF-α (d) and TGF-β1 (e) are surveyed by RT-PCR.

Figure 1-4. Effect of gel-type CM-chitin on expression of TGF-β1 in PECs. Total PECs were harvested from mice at 24 h after administration of gel-type CM-chitin, β-TCP, CM-chitin/β-TCP composite or PBS cultured for 24 h, and their supernatants were used for ELISA to evaluate the expression of TGF-β1. Each value is the mean ± SE of 3 samples. ** indicate the significances at $p<0.01$ compared with β-TCP treated group. ## indicates the significance at $p<0.01$ compared with CM-chitin/β-TCP composite treated group.

Figure 1-5. Effect of soluble-type CM-chitin on expression of TGF-β1 in fractionated PECs. PECs were harvested from mice at 24 or 48 h after administration of CM-chitin or PBS and fractionated by density gradient. The MNC fractions were cultured for 24 h and their supernatants were used for ELISA to evaluate the expression of TGF-β1. Each value is the mean ± SE of 3 samples. * and ** indicate the significances at $p<0.05$ and $p<0.01$, respectively, compared with PBS treated group. # indicates the significance at $p<0.05$, compared with soluble-type CM-chitin treated group at 24 h.

Figure 1-1

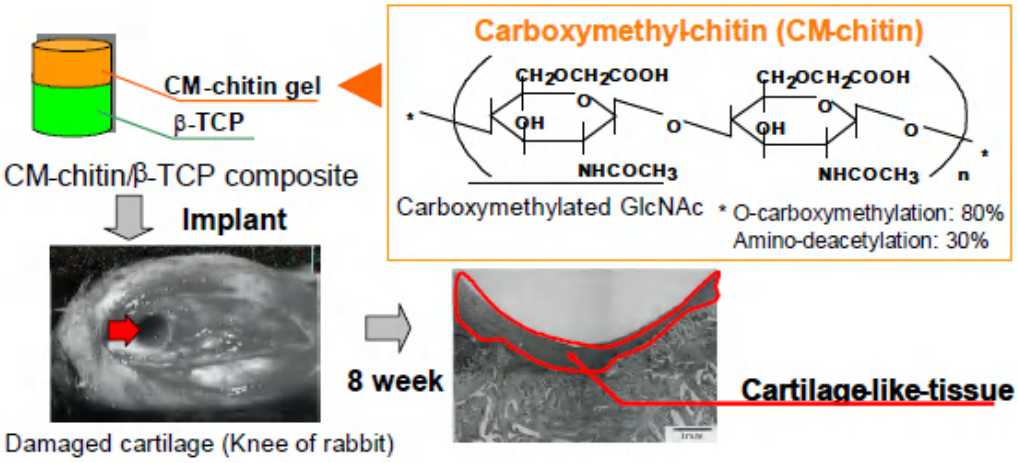


Figure 1-2

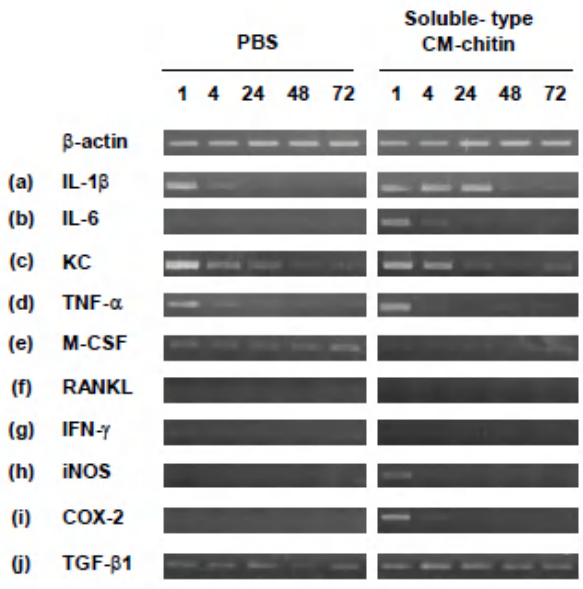


Figure 1-3

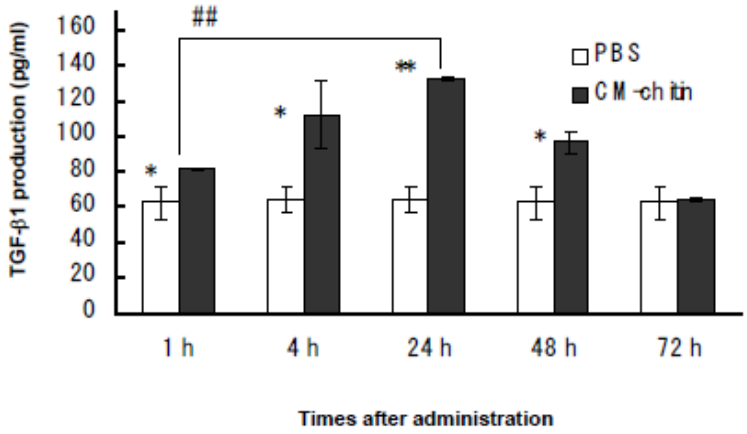


Figure 1-4

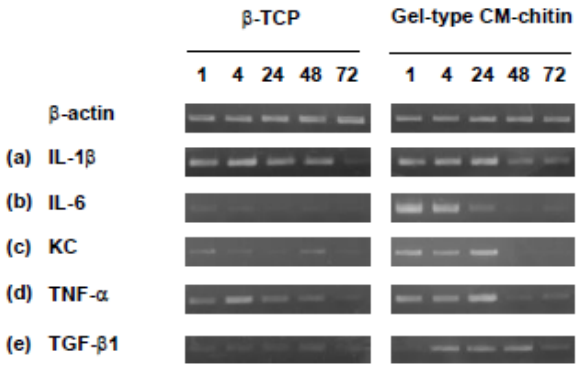


Figure 1-5

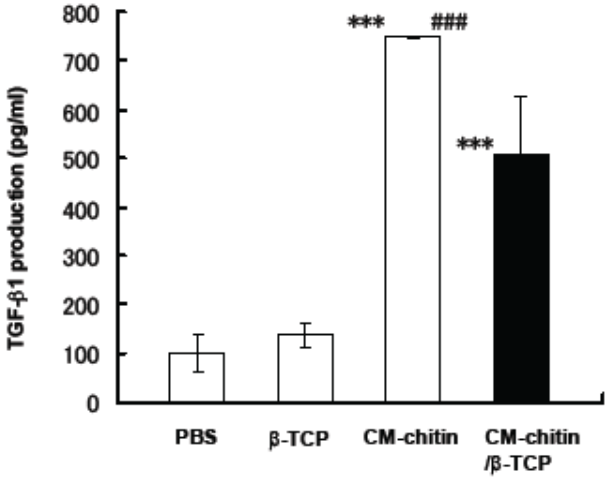
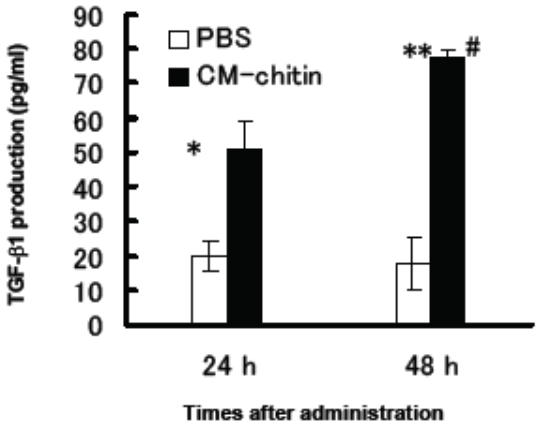


Figure 1-6



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Section 2.

Carboxymethyl-chitin promotes chondrogenesis by inducing the production of growth factors from immune cells.

2-1. Abstract

Many techniques have been tested for their ability to restore cartilage defects, but several problems still remain in the complete healing of injured cartilage. In our previous study, we found that a carboxymethyl-chitin / β-tricalcium phosphate (CM-chitin/β-TCP) composite induced cartilage regeneration in the osteochondral defects of rabbits *in vivo*. We also found that CM-chitin stimulated peritoneal exudate cells (PECs) in mice and induced several kinds of inflammatory cytokines and transforming growth factor beta-1 (TGF-β1).

In this study, we examined whether CM-chitin is responsible for the induction of chondrogenesis via the production of TGF-β1 *in vitro*. The murine pluripotent cell line C3H10T1/2 was maintained as a micromass culture in conditioned medium prepared from PECs stimulated with and without CM-chitin. CM-chitin conditioned medium induced RNA expression of the chondrogenic-factor Sox9 and the matrix proteins aggrecan, Col2a1, and Comp. Their expression levels were decreased in the presence of anti-TGF-β1 antibody. The micromass tissues cultured in CM-chitin conditioned medium at day 21 were clearly stained by Toluidine blue or Alcian blue (histological staining) and collagen II antibody (immunohistological staining), showing the expression of acidic glycosaminoglycan and type II collagen. Similar results were observed in micromass tissue stimulated with TGF-β1 as a positive control. However, no chondrogenesis occurred when CM-chitin was added directly to a C3H10T1/2 cell culture. These results indicated that CM-chitin is a potent inducer of chondrogenesis via the induction of TGF-β1 in immune cells.

2-2. Introduction

Cartilage is known to have a poor capacity for self-repair because of its low mitotic activity and avascular nature [1,2]. Several medical and surgical methods have been tested for their ability to repair cartilage defects, but complete regeneration of normal cartilage in damaged areas is difficult to achieve due to several problems such as the loosening of artificial joints, degenerative changes in transplanted tissues [3], and the risk of viral transmission [4,5].

Recently, tissue-engineering approaches using biocompatible scaffolds have been applied to the treatment of cartilage defects [4,6-9], and many materials such as collagen [10], hydroxyapatite [11,12], polyglycolic acid [13], hyaluronic acid [14,15], and silk [6,7] have been investigated. Such scaffolds play an important role in promoting the formation of cartilage as a temporary extracellular matrix (ECM). In many cases, the scaffold-based tissue-engineering technique requires the addition of growth factors, such as transforming growth factor-beta (TGF-β) or bone morphogenetic protein (BMP). It is well known that these growth factors promote the proliferation or differentiation of chondrocytes from mesenchymal stem cells *in vivo* [16-19] and *in vitro* [4,7,20,21]. Although tissue-engineering techniques that combine scaffolds with growth factors have great potential for cartilage repair, they are still at an experimental stage because of the cost of using a large amount of expensive recombinant growth factors.

In our previous study, we found that a carboxymethyl-chitin (CM-chitin)/β-tricalcium phosphate (β-TCP) composite induced the regeneration of cartilage in the osteochondral defects of rabbits (Figure1-1) [22]. The regeneration of cartilage was observed after treatment with the CM-chitin/β-TCP composite but not with β-TCP alone eight weeks after implantation, indicating that CM-chitin plays a key role in the regeneration of cartilage. Previously, we also showed that CM-chitin stimulated murine peritoneal exudate cells (PECs) to induce the expression of inflammatory cytokines and TGF-β1 [23]. TGF-β1 has many functions [24], including the induction of mesenchymal cell condensation, which is required for *in vitro* chondrogenesis [25, 26, 27]. However, no correlation between the stimulation of cells induced with CM-chitin and the regeneration of cartilage has been demonstrated.

The murine pluripotential cell line C3H10T1/2 has been shown to

differentiate into myoblasts, osteoblasts, adipocytes, and chondrocytes after treatment with growth factors [20]. C3H10T1/2 cells are attractive for studying chondrogenesis because they do not spontaneously differentiate under standard culture conditions. Previously, treatment of a high-density micromass culture of C3H10T1/2 cells with TGF-β1 was reported to induce the formation of a three-dimensional spheroid structure and chondrogenic differentiation [20, 28-30]. In this study, we examined the effect of CM-chitin on chondrogenesis using the above mentioned *in vitro* micromass culture system.

2-3. Materials and methods

2-3-1. *CM-chitin preparation and cell stimulation*

The CM-chitin (degree of substitution for O-carboxymethylation = 79 mol%, degree of deacetylation = 27 mol%) was prepared from chitin extracted from Queen Crab shells, according to a previously reported method [31]. Gel-type CM-chitin was prepared as described [22] and suspended in PBS (10 mg/ml). The level of endotoxin contamination was determined by the Endospecy® test (Seikagaku Biobusiness, Tokyo, Japan) according to the manufacturer's instructions.

In this experiment, we used a PECs-utilizing micromass culture as an experimental model of chondrogenesis. Eight to ten-week-old male BALB/cN-sea mice were obtained from Kyudo (Saga, Japan). The animal experiment was performed in accordance with our institutional approval (H18Eng007) and guidelines, and the legal requirements of Japan. The mice were injected intraperitoneally (ip) with 0.5 ml of PBS or CM-chitin gel suspension (1 mg/ml). After 24 h stimulation, the ip-administered mice were sacrificed, and 5 ml PBS were injected into the peritoneal cavity. After extracting peritoneal exudate fluid, we collected PECs from the fluid. The number of cells was 1-3 × 10^6 cells per mouse. After 24 h culture in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS, Equitech-Bio, Kerrville, Texas, USA), 100 unit/ml penicillin, and 100 μg/ml streptomycin, the supernatants were collected and subjected to ultra-filtration with Amicon Ultra-15 Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The supernatants were concentrated to a 1/45 volume and used as PBS conditioned medium (PBS-sup) and CM-chitin conditioned medium (CM-chitin-sup), respectively (Scheme1).

2-3-2. *Monolayer culture of C3H10T1/2 cells*

The murine pluripotential cell line C3H10T1/2 (Health Science Research Resources Bank, Tokyo, Japan) was maintained as a monolayer culture in 25 or 75 cm^2 polystyrene tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ, USA) in DMEM supplemented with 10% FBS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated in a humidified incubator at 37°C and 5% CO_2, and the culture medium was changed every 3 days.

C3H10T1/2 cells were seeded on 24-well culture plates at 10^5 cells/well and cultured with PBS (10% in medium), CM-chitin solution (1 mg/ml), CM-chitin gel suspension (1 mg/ml), or recombinant TGF-β1 (10 ng/ml, PeproTecch, NJ, USA) for 1-5 days. As a negative control, Triton X-100 was added to lyse the cells. The cell culture supernatants were collected every day and used in a cytotoxicity detection assay with a lactate dehydrogenase (LDH) assay kit (Roche Applied Science, Mannheim, Germany) in accordance with manufacturer's procedure. The viability of the remaining cells was determined by 0.5%-Trypan blue staining of cells treated with trypsin before they were scraped off the wells. Data are expressed as the mean ± SE of three independent experiments. The LDH values and cell numbers of each group were analyzed for statistical significance using Welch's t-test. Total RNA was extracted from the cells at 7, 14, and 21 days, and the mRNA expression of growth factors and cartilage-related genes was analyzed using the reverse transcriptase polymerase chain reaction (RT-PCR) as described previously [23]. The pairs of primer sequences used were as follows: For β-actin: 5'- ATGGATGACGATATCGCT -3' (sense) and 5'- ATGAGGTAGTCTGTCAGGT -3' (antisense). For TGF-β1: 5'- TACTATGCTAAAGAGGTCACCC -3' (sense) and 5'- TCCTTGGTTCAGCCACTGCC -3' (antisense). For Agr: 5'- AGTGGATCGGTCTGAATGACAGG -3' (sense) and 5'- AGAAGTTGTCAGGCTGGTTTGGA -3' (antisense). For Col2a1: 5'- AGGGCAACAGCAGGTTCACATAC -3' (sense) and 5'- TGTCCACACCAAATTCCTGTTCA -3' (antisense).

2-3-3. *Micromass culture*

The micromass culture experiment was performed according to the protocol of Ahrens et al. [32] with slight modifications (Figure 2-1). In brief, the C3H10T1/2 cells were trypsinized and resuspended in DMEM medium containing 10% FBS (basic medium) at a concentration of 2 x 10^7 cells/ml, and 10 μl of the suspension were transferred to a 48-well culture plate and allowed to adhere for 1 h at 37°C in 5% CO_2. After the addition of 80 μl of basic medium and 20 μl of PBS, recombinant TGF-β1 (10 ng/ml), or one of the conditioned media (PBS-sup or CM-chitin-sup), the cells were cultured at 37° C in 5% CO_2. The medium was changed every 4 days, and micromass tissues were collected at 7, 14, 21, and 28 days. For the TGF-β1 blocking experiment,

anti-TGF-β1 mAb (R&D systems, Tokyo, Japan) were added in TGF-β1 medium or CM-chitin-sup at 10 μg/ml and incubated for 1 h at room temperature before being used for the micromass culture. Each collected micromass tissue was used for mRNA expression analysis or histological staining.

2-3-4. RNA extraction, cDNA synthesis, and PCR analysis

The total cellular RNA was extracted from the micromass tissue. RT-PCR was performed using the SideStepTM II QPCR cDNA Synthesis Kit (Stratagene, CA, USA), and consecutive quantitative polymerase chain reactions (Q-PCR) were performed using SYBR Premix Ex Taq (Takara Bio, Shiga, Japan), according to the manufacturer's procedure. The sense and anti-sense primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aggrecan (Agr), collagen-2a1 (Col2a1), cartilage oligomeric matrix protein (Comp), sex-determining region Y-box9 (Sox9), collagen-1a1 (Col1a1), and collagen-10a1 (Col10a1) were purchased from Takara Bio (Shiga, Japan). The amplified products were analyzed using the 7300 Real-Time PCR System (Applied Biosystems, CA, USA), and the transcription level normalized to GAPDH was then calculated using the 2^{Δ} Ct formula with reference to the undifferentiated mesenchymal stem cells.

2-3-5. Histology and Immunohistology

After 21 and 28 days, the collected micromass tissues were rinsed with PBS, fixed with 4% formaldehyde for 30 min, and washed twice with PBS. The tissues cultured in medium containing nothing, TGF-β1, PBS-sup, or CM-chitin-sup were collected on day 21 and stained with Alcian blue (pH 1.0, 1% Alcian blue 8 GX [Sigma-Aldrich, MO, USA] in 0.1 M HCl) overnight at 4˚C and washed with PBS.

The tissues cultured in TGF-β1 or CM-chitin-sup until day 28 were embedded in paraffin, sectioned at 12 μm using a microtome, and deparaffinized. The tissues cultured in medium alone or with PBS-sup until day 28 were used directly for staining because they did not form 3-D aggregates. One set of samples was stained by 0.05% Toluidine blue (pH7.0) before being washed with 99% EtOH. At the same time, the other set of samples was subjected to Protease K treatment and blocking treatment and incubated with Rabbit anti-mouse Collagen II polyclonal-antibody (ab21291,

Abcam Japan, Tokyo, Japan) for 30 min. After being washed with TBS, the samples were incubated with the secondary antibody (EnVision™+ Rabbit/HRP, DAKO Japan, Kyoto, Japan) before being washed with TBS. The samples were then stained with a substrate (DAB+ Liquid, DAKO Japan) for 1 min and washed with water, before being observed with an IX70 microscope (Olympus Medical Systems, Tokyo, Japan).

2-4. Results

2-4-1. Effect of CM-chitin on a monolayer culture of C3H10T1/2 cells

First, we checked the endotoxin level of CM-chitin using the Endospecy® test. As a result, only a small amount of endotoxin was detected in gel-type CM-chitin at 100 μg/ml.

We then investigated the effects of CM-chitin on cell viability, proliferation, and stimulation. The LDH values showed that neither CM-chitin nor CM-chitin gel affected the cell viability of C3H10T1/2 cells and that there was no significant difference between the two treatments (Figure2-2a). The proliferation rates of the C3H10T1/2 cells treated with PBS, CM-chitin, and CM-chitin gel were similar during the culture period, while the proliferation rate of the cells treated with TGF-β1 was higher than those of the cells treated with other reagents, especially at 3 – 5 days of culturing (Figure1b). The RNA expression of growth factors and two cartilage-related genes in the C3H10T1/2 cells in monolayer culture was not significantly different among the PBS, CM-chitin, CM-chitin gel, and TGF-β1 treated groups (Figure2-3).

2-4-2. Expression of chondrogenic markers in micromass culture

To examine whether CM-chitin-sup, a conditioned medium from PECs stimulated with CM-chitin gel, promotes chondrogenesis in micromass culture, the mRNA expression of six chondrogenic markers in micromass tissues was quantified by Q-PCR. The mRNA expression of Agr and Col2a1 was significantly increased in the CM-chitin-sup treated samples on day 21, as well as in the TGF-β1 treated samples (Figure2-4a,b). In the CM-chitin-sup treated micromass culture, the expression of Agr was found to be diminished on day 28, while the expression of Col2a1 was further increased on day 28 (Figure3a,b). Sox9 and Comp mRNA were highly expressed on day 7 (Sox9) and 14 (Comp), but no expression was observed on day 28 (Figure2-4c,d). Relatively high expression of Col1a1 and Col10a1 was also observed on days 21 and 28, respectively (Figure2-4e).

mRNA expression related to chondrogenesis was blocked by anti-TGF-β1 antibody (Figure2-5). The Agr, Co2a1, and Sox9 expression levels in the CM-chitin-sup and TGF-β1 treated micromass cultures were significantly decreased in the presence of the antibody.

2-4-3. Histological analysis of micromass culture

To examine whether the growth factors secreted from PECs stimulated with CM-chitin induce chondrogenesis, the accumulation of chondrogenesis-related ECM in the micromass tissues was investigated by staining with Alcian blue, Toluidine blue, and anti-Collagen II antibody. The specific aggregation of micromass tissues was observed in the TGF-β1 treated micromass culture and CM-chitin-sup treated micromass culture (Figure5a). However, neither the non-treated micromass culture nor the PBS-sup treated micromass culture formed 3-D aggregates.

In the presence of TGF-β1 or CM-chitin-sup, the tissues showed a strong blue color under Alcian blue staining (Figure2-6a,b) and a magenta color under Toluidine blue staining (Figure2-6c), indicating the presence of sulfated glycosaminoglycan [33]. Immunohistological staining also showed the presence of collagen II in these micromass tissues (Figure2-6d). The micromass tissues collected on day 7 or 14 were also subjected to staining, but no marked staining was observed (data not shown). In contrast, the tissues treated with nothing or PBS-sup showed a weak blue color under Alcian blue staining and a blue color under Toluidine blue staining, indicating the absence of chondrogenesis-related ECM.

2-5. Discussion

We previously reported that CM-chitin induced the regeneration of hyaline cartilage in rabbits *in vivo* [22]. In contrast to conventional scaffold-based tissue-engineering methods using growth factors, no growth factor is required in our CM-chitin-based method, suggesting that CM-chitin has dual functions, as a scaffold and an inducer of cartilage-differentiation. We therefore investigated whether CM-chitin directly affects the cartilage differentiation of progenitor cells. The murine pluripotent cell line C3H10T1/2 was used in the present study. CM-chitin did not significantly alter the viability or proliferation of the cells (Figure2-2a,b), similarly to other biomaterials, such as polylactic acid [34], polylactic acid - polyglycolic acid [35], poly(DL-lactic-co-glycolic acid) [36], and silk [7,37]. Furthermore, CM-chitin did not influence the expression of growth factor or chondrogenic markers in C3H10T1/2 cells (Figure2-3). These results indicate that CM-chitin did not contribute directly to the differentiation of pluripotent cells into chondrocytes.

We recently found that CM-chitin stimulates immune cells to produce TGF-β1, a growth factor for cartilage regeneration [23]. However, it was unclear whether the stimulation of cells by CM-chitin leads to cartilage regeneration. We demonstrated here that the factors secreted from cells stimulated with CM-chitin induced the expression of chondrogenic markers. Micromass tissue of C3H10T1/2 cells, cultured in the presence of CM-chitin-sup, expressed mRNA of chondrogenic markers including aggrecan and type II collagen (Figure2-4).

Agrrecan (Agr), type II collagen (Col2a1), and Sox9 are in vivo and in vitro markers of chondrogenesis. Agr is an integral part of ECM components in cartilage [38-41], and its expression is induced by several factors including TGF-β1, TGF-β3, BMP-2, and dexamethasone in micromass culture, pellet culture, or an appropriate three-dimensional culture [12,43-46]. TGF-β1 is reported to promote the expression of Col2a1 and Sox9 [47]. Sox9, a transcription factor, acts during chondrocyte differentiation and activates the transcription of Agr and Col2a1 [40,48,49]. Type II collagen, which includes an alpha-1 chain encoded by Col2a1, is the most abundant and important component of the chondrogenesis-related ECM, and mutations in this gene are associated with achondrogenesis and chondrodysplasia [50,51].

It has also been shown in previous studies that the expression of Col2a1 was accompanied by an increase in Agr or glycosaminoglycan expression during chondrogenesis [52-54]. The time-courses of the expression of Sox9, Col2a1, and Agr in the present study (Figure2-4a,b,c) were coincident with the results of the above reports. In our results, the expression of Agr was decreased in both the TGF-β1 treated micromass culture and CM-chitin treated micromass culture at day 28 (Figure2-4a); whereas, Col2a1 expression was increased in the CM-chitin treated micromass culture while its expression was decreased in the TGF-β1 treated micromass culture at day 28 (Figure2-4b). Previous research of murine limb-bud-cell micromass culture reported that the high expression of Col2a1 decreases gradually and that the expression of collagen10, which is commonly observed in hypertrophic cartilage, is replaced by Col2a1 expression [27]. In our experiment, the Col2a1 and Col10a1 expression in TGF-β1 treated micromass culture seemed to correspond with above mentioned report (Figure2-4b,f). On the other hand, the Col2a1 and Col10a1 expression in the CM-chitin treated micromass culture did not. The differences in Col2a1, Comp, and Col1a1 expression between the CM-chitin treated and TGF-β1 treated micromass cultures (Figure2-4b,d,e) were probably due to another factor secreted from PECs, but this remains to be elucidated. We also showed here that blocking the TGF-β1 signal with anti-TGF-β1 antibody inhibited the expression of chondrogenic markers (Figure2-5a-c). This indicated that TGF-β1 is one of the most important factors for chondrogenesis in micromass culture using CM-chitin-sup.

It has also been shown in previous studies that Comp, cartilage-oligomeric-protein, binds to collagen type I and II in the ECM and facilitates their structural stability [55.56]. While Comp is synthesized by chondrocytes, osteoblasts, tenocytes, and ligament cells *in vivo* [57,58], it is also synthesized by several mesenchymal cell-derived chondrocytes *in vitro* [49,59-60]. It was also reported that several types of TGF-β promote the expression of Comp in chondrogenic tissue from MSC [61]. Our result, which showed enhancement of Comp expression in micromass tissue due to treatment with TGF-β1 or CM-chitin-sup, is consistent with this report.

Histological evaluation showed the induction of glycosaminoglycan and type II collagen in CM-chitin-sup treated micromass tissue (Figure2-6). Furthermore, a specific aggregation pattern was observed in the micromass

tissue (Figure2-6a). It has been reported that the treatment of micromass cultures of C3H10T1/2 cells with TGF-β1 resulted in the formation of a three dimensional spheroid culture exhibiting cartilage-like histology [28,30]. The aggregation pattern of the CM-chitin-sup treated micromass tissue closely resembled that of the TGF-β1 treated micromass tissue (Figure2-6a), suggesting that the factors secreted by cells stimulated with CM-chitin induced chondrogenesis in micromass culture.

Our results obtained in the present and previous studies suggested that the stimulation of CM-chitin induces the secretion of TGF-β1 from immune cells and results in the promotion of chondrogenesis in micromass culture. This shows that CM-chitin plays dual roles in cartilage regeneration as a scaffold and growth factor inducer. Recently, Yasuda et al. developed a novel method for inducing spontaneous *in vivo* cartilage regeneration by implanting a double-network hydrogel material into osteochondral defects of the femoral joint [62]. They observed spontaneous *in vitro* and *in vivo* chondrogenesis using a functional polymeric compound. Here, we also observed spontaneous *in vitro* chondrogenesis with a CM-chitin polymer without using any additional growth factors. Although the mechanism of the induction of TGF-β1 production in immune cells treated with CM-chitin has not been clarified, the CM-chitin-based cartilage regeneration method is a good candidate for tissue-engineering as it does not require growth factors.

2-6. Figures and figure captions

Figure 2-1. A procedure for micromass culture. The conditioned media were prepared separately and applied to micromass culture.

Figure 2-2. Cytotoxic and proliferative assay for CM-chitin. a) Cytotoxicity was evaluated with a lactate dehydrogenase (LDH) assay. Each LDH value represented the cytotoxicity of the respective reagent. The cells in the triton group were disrupted by triton reagent, so the LDH-data of this group was used as a negative control. b) Cell proliferation was determined by cell counting using a hemocytometer from days 1 to 5.

Figure 2-3. Effect of CM-chitin on monolayer culture of C3H10T1/2 cells. The cells were cultured as a monolayer in the presence of the indicated stimuli for 21 days. mRNA was obtained from the cells in each well and subjected to RT-PCR for the detection of TGF-β1: transforming growth factor-β1, Agr: aggrecan, and Col2a1: collagen2a1.

Figure 2-4. Quantitative mRNA expression of cartilage-related genes in micromass tissues. Micromass tissues were treated with TGF-β1 (10 ng/ml), PBS-conditioned medium (PBS-sup), or CM-chitin conditioned medium (CM-chitin-sup) for 7, 14, 21, or 28 days, and their gene expression was compared to that of non-treated micromass tissue (NC). Data are expressed as the mean ± SE of 3 independent experiments. mRNA expression was normalized to that of GAPDH.

Figure 2-5. TGF-β1 blocking experiment using anti-TGF-β1 antibody. Micromass tissues were treated with TGF-β1 (TGF, 10 ng/ml), anti-TGF-β1 antibody + TGF-β1 (a-TGF/TGF, a-TGF = 10 μg/ml), CM-chitin conditioned medium (CM-chitin-sup), or anti-TGF-β1 antibody + CM-chitin conditioned medium (a-TGF/CM-chitin-sup, a-TGF = 10 μg/ml), and their gene expression was compared to that of non-treated micromass tissues (NC). Micromass tissues were used for QPCR analysis of aggrecan (Agr), Collagen-2a1 (Col2a1), and Sox9 at the highest expression point of each gene (at day 21, 21, and 7, respectively). Data are expressed as the mean ± SE of 3 independent

experiments. mRNA expression was normalized to that of GAPDH.

Figure 2-6. Histological evaluation of micromass tissues. a) Alcian blue staining of tissues on day 21, observed at low magnification. b) Alcian blue staining of tissues on day 21, observed at high magnification. c) Toluidine blue staining of tissues on day 28. Tissues containing acidic glycosaminoglycan were stained a deep magenta color. d) Immunohistological staining of tissues on day 28 with anti-collagen II antibody. Tissues containing collagen II were stained deep brown. In Figures c) and d), samples of TGF-β1 medium (TGF) and CM-chitin conditioned medium (CM-chitin-sup) were paraffinized, sectioned (12 μm), and de-paraffinized before staining, while non-treated (NC) and PBS conditioned medium (PBS-sup) treated tissues were not subjected to sectioning.

Figure 2-1

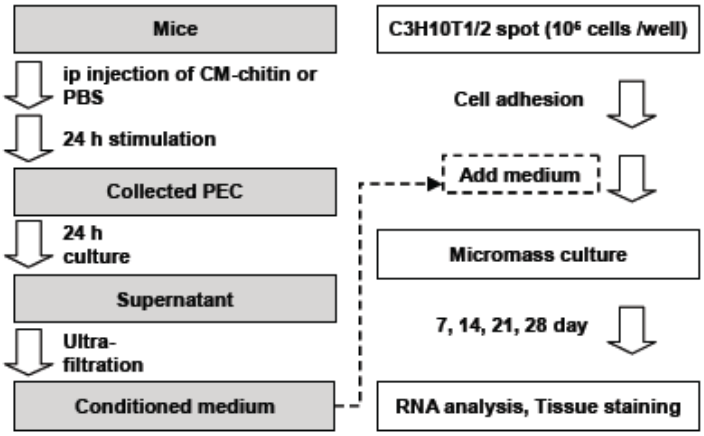


Figure 2-2

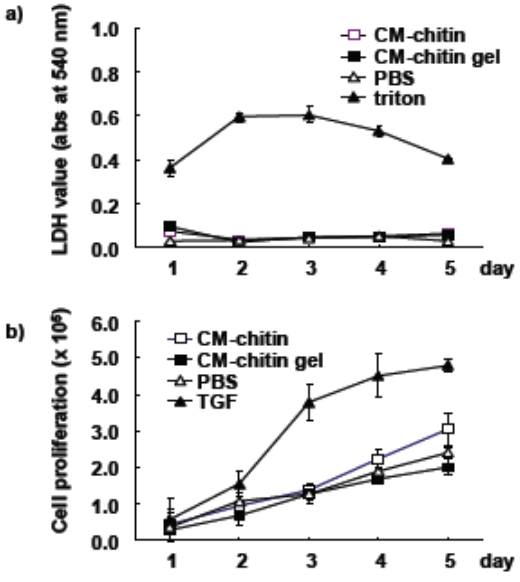


Figure 2-3

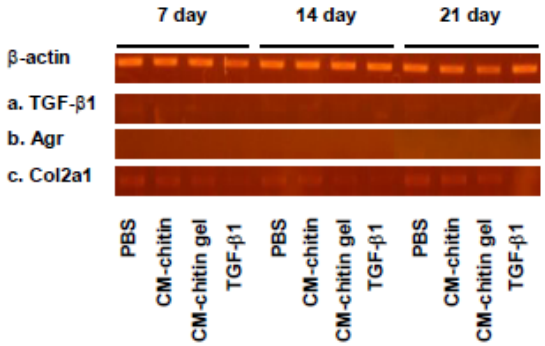


Figure 2-4

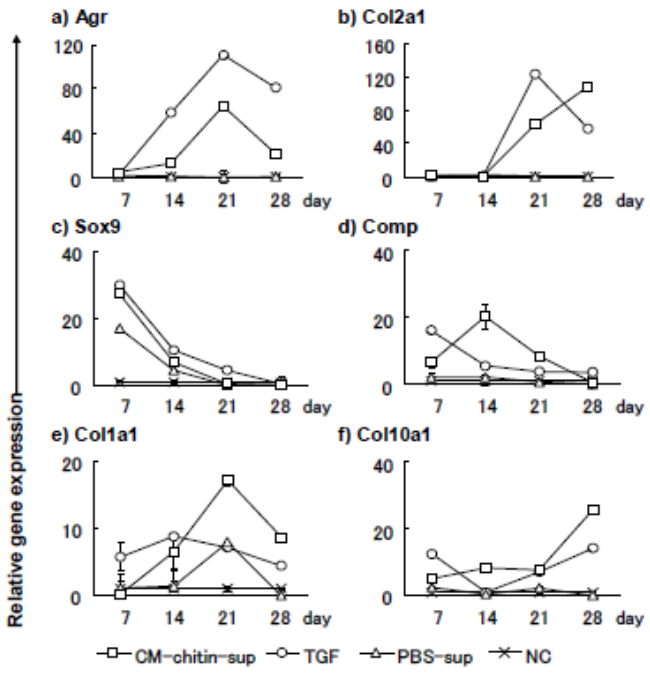


Figure 2-5

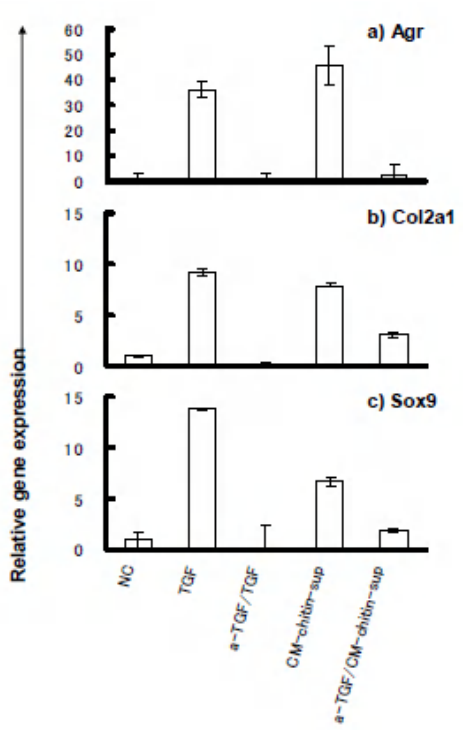
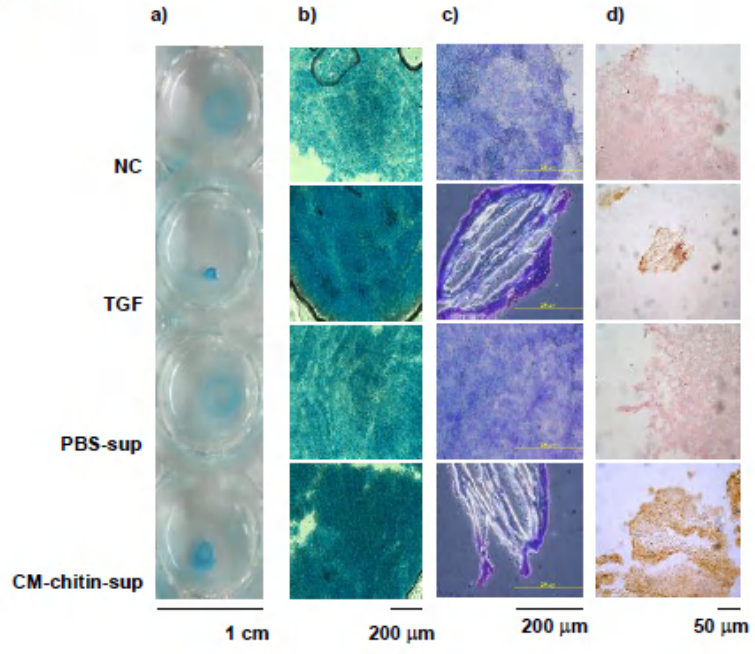


Figure 2-6



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Section 3.

Enhancement of bactericidal activity of phagocyte by carboxymethyl-chitin.

3-1. Abstract

Previously we found that carboxymethyl-chitin (CM-chitin) / β-tricalcium phosphate (β-TCP) composite induced regeneration of cartilage in rabbit *in vivo*. We also reported that CM-chitin induced to secrete several inflammatory cytokines. However, roles of those cytokine secretions have not yet been identified. Here, we showed that CM-chitin induced phagocytes recruitment and enhanced their bactericidal activity.

Peritoneal exudate cells (PECs) were collected from Balb/c mice, which were beforehand administrated ip with CM-chitin and FITC-labeled *Escherichia coli* or *Staphylococcus aureus* at 24 h, and analyzed by flow cytometry. Mouse macrophage J774A.1 and human monocytic THP1 were incubated with CM-chitin for 4 or 24 h. Reactive nitrogen and oxygen species (RNS and ROS) in the supernatant were measured by Griess and WST-1 method, respectively. The cells were also preincubated with CM-chitin and further incubated with *E. coli* or *S. aureus* for 4 or 24 h. Viability of bacteria, which was phagocytized in the cells, was evaluated by thiazole-orange / propidium iodide (TO/PI) staining followed by flow cytometry analysis.

CM-chitin administration into mice notably increased the number of PECs and enhanced phagocytosis activity of PECs, which were mainly consisted by macrophage. CM-chitin stimulation also increased production of RNS/ROS in the two cell lines. TO/PI staining of bacteria showed that mortality rate of cell-internalized bacteria was significantly higher in J774A.1 treated with CM-chitin than in the cell with other treatments. These results suggested that CM-chitin augmented bactericidal activity through the increase of phagocytes and the enhancement of their phagocytosis activity and their RNS production.

3-2. Introduction

Chitin, β-(1-4)-poly-N-acetyl-D-glucosamine, is the second most abundant biopolymer in nature after cellulose. Chitin is known as a cell wall component of bacteria, fungi and an exoskeleton of crustaceans and insects (1-5). Chitin is used by these organisms to protect from inclement conditions in their environment and host immune responses (5). Because of the good biocompatibility and biodegradability, chitinous derivatives have been applied in food, agriculture, textile, polymers, wastewater treatment and medical applications (6-7). In the medical application, chitinous derivatives have been reported to exhibit adjuvant activity (8-9), antitumor activity (10), wound-healing acceleration activity (11-12), antimicrobial and antiviral activity and stimulating activity of immune response (13-15).

Recently, immunobiological functions of chitinous derivatives have been reported. Chitin and chitosan caused complementary activation via an alternative pathway (16-17), chitin stimulates several pattern recognition receptors in a size-dependent manner (18-20), and chitin is associated to allergic immunity and induces alternative activation of macrophage (21-22). These results suggested that chitin derivatives are able to modulate immune systems.

Low solubility of chitin is considered to be a main limiting factor for its application and carboxymethyl chitin (CM-chitin) is one of the water-soluble forms of chitin (23). CM-chitin has been extensively applied in agricultural, medical or pharmaceutical fields (24-26). It has also been reported that CM-chitin induced cytotoxic macrophages and the function should be dependent on the degree of carboxymethylation (27, 28). However, more detailed analysis for immunological function of CM-chitin has not been reported.

Previously we found that composite of CM-chitin and □-tricalcium phosphate (β-TCP) induced regeneration of cartilage in osteochondral defect of rabbits (26). We recently demonstrated that CM-chitin stimulated murine peritoneal exudate cells (PECs) to induce the expression transforming growth factor β1 (TGF-β1) (30) and the TGF-β1 promoted *in vitro* chondrogenesis from murine pluripotential cell (31). These data indicated that CM-chitin is responsible for cartilage regeneration. We also reported that CM-chitin induced inflammatory cytokines in murin PEC (30). These

results suggested that CM-chitin modulated immune response, but the system is not yet clearly defined. In this paper, we aimed to assess the antimicrobial activity of CM-chitin.

3-3. Materials and methods

3-3-1. *CM-chitin preparation*

The CM-chitin (degree of substitution for O-carboxymethylation = 79 mol%, degree of deacetylation = 27 mol%, molecular weight = 5 x 10^5) was prepared from chitin extracted from Queen Crab shells, according to a previously reported method and CM-chitin used for this experiment was prepared as described [23]. Briefly, CM-chitin was dissolved in Milli-Q water, precipitated in EtOH, and the precipitate was then processed by heat treatment in a vacuum (approximately 1.0-1.5 kPa) at 140°C for 12 h. The product was sterilized by Co-60 γ-ray irradiation (25 kGy) and diluted to 10 mg/ml by phosphate buffer saline (PBS). PBS or beta-tricalcium phosphate (β-TCP) was used for negative control in experiment. Endotoxin level of CM-chitin was checked by Endospecy® test (Seikagaku Biobusiness, Tokyo, Japan) according to the manufacturer's instructions and trace amount of endotoxin was detected in gel-type CM-chitin even at 100 μg/ml (data not shown).

3-3-2. *Cells, Bacteria and fluorescent-particles*

The animal experiment was performed in accordance with our institutional approval (H18Eng007) and guidelines, and the legal requirements of Japan. Eight to ten-week-old male Balb/cN-sea mice (Kyudo Co. Ltd., Saga, Japan) were injected intraperitoneally (ip) with 0.5 ml of PBS, CM-chitin gel suspension (10 mg/ml) or thioglycolate broth. After 24 h stimulation, the mice were sacrificed and the peritoneal cavity was washed with 5 ml PBS. To collect peritoneal exudate cells (PECs). Splenic macrophages were also isolated from Balb/cNsea mice. Murine-derived macrophage cell line J774A.1 and human-derived monocyte cell line THP1 were purchased from Health Science Research Resources Bank (Osaka, Japan). J774A.1 and THP1 were cultured in Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI1640) medium, respectively, supplemented with 10% fetal bovine serum (FBS, Equitech-Bio, Kerrville, Texas, USA) and 1% penicillin-streptomycin (PS, 10000 units/ml and 10000 μg/ml, respectively). All mammalian cells were cultured in 5% CO_2 incubator at 37°C.

Staphylococcus aureus (lineage DSM20634) and Escherichia coli

(lineage JCM1648) were cultured in brain-heart-infusion (BHI) medium. Fluorescein isothiocyanate (FITC)-labeled latex-beads (F-Bs) or *S aureus* (F-Sa) and *E coli* (F-Ec) were purchased from Polysciences Inc. (USA) or Invitrogen Corp. (USA).

3-3-3. *Stimulation of PECs by CM-chitin*

Mice were administered ip with CM-chitin or β-TCP (5 mg/mouse). After 24 h stimulation, the mouse was again injected ip with labeled-particles (F-Bs, F-Sa or F-Ec, each 8 x 10^6/mouse). PECs were collected from mice at 4 h after administration of particles, and washed with PBS and filtered by cell strainer (70 μm, BD Falcon, USA). Total numbers of PECs were counted by hemocytometor.

The PECs were treated with anti-mouse CD16/32 monoclonal abtibody (mAb) for Fcγ receptor blocking (eBioscience, USA) at optimal concentration on ice for 10 min, and then stained with PE/Cy7-conjugated anti-mouse F4/80 mAb (BioLegend, USA) and PE/Cy5-conjugated anti-mouse Gr1 mAb (eBioscience,) on ice under the dark condition for 30 min. The PECs were washed with PBS for 2 times, fixed with 4% formaldehyde-PBS, and analyzed by flow cytometer (FC500, Beckman Coulter).

$F4/80^+Gr1^+$ cells and $F4/80^-Gr1^+$ cells were considered as macrophages and neutrophils, respectively. Rate of cells engulfing fluorescent-labeled microparticles was estimated by FITC intensity. Statistical significance among the cell numbers in several experimental groups was analyzed by Student's t-test.

3-3-4. RNA extraction, cDNA synthesis, and Q-PCR analysis

Total RNA was extracted from PECs or J774A.1, which were stimulated with CM-chitin, PBS or β-TCP for 1 to 24 h, by use of TRIzol® Reagent (Invitrogen Inc., USA) and subjected to RT-reaction by use of RNA PCR Kit (AMV) Ver.3.0 (TaKaRa BIO Inc., Japan) according to the manufacturer's procedure. Q-PCR were performed by use of SYBR Premix Ex Taq (Takara Bio, Japan) and 7300 Real-Time PCR System (Applied Biosystems, USA), according to the manufacturer's procedure. The sense and antisense primers for monocyte chemotactic protein-1 (MCP1), keratinocyte chemoattractant (KC) and induced-NO synthesis gene (iNOS) were

purchased from Sigma (Japan). Their sequences are as follows. MCP1: sense is "CCTGGATCGGAACCAAATGA" and antisense is "CGGGTCAACTTCACATTCAAAG", KC: sense is "CCTTGACCCTGAAGCTCCCTTGGTTC" and antisense is "CGTGCGTGTTGACCATACAATATG", iNOS: sense is "TCACTGGGACAGCACAGAAT" and antisense is "TGTGTCTGCAGATGTGCTGA". The transcription level normalized to GAPDH was calculated using the 2^{Δ} Ct formula with reference to the stimulated control cells.

3-3-5. *RNS, ROS production from phagocyte*

J774A.1 or THP1 suspended in FBS/PS-free RPMI was placed on 96-well plate at 3 x 10^8 cells/well. After 1h incubation, the cells were stimulated with 2 mg/ml CM-chitin and co-stimulator, latex-beads. The cells were cultured for 4 h or 24 h, and then supernatants were subjected to RNS/ROS detection. For RNS detection, the supernatant was mixed with Griess reagent (consist of 1% sulfanilamide in 5% phosphoric acid solution and 0.1% Naphthylethylen diamine dihydro-chloride) and followed by the measurement of optical density at 540 nm. Standard curve for RNS concentration was based on the sequential dilution of sodium nitrite. For ROS detection, WST-1 reagent (2-(4-Iodophenyl)-3-(4-nitrophenyl) -5-(2,4-disulfophenyl)-2H-tetrazolium) was mixed in culturing medium (at 250 μM, final concentration). After 24 h incubation, optical density of the supernatant was measured by microplate reader at 450 nm. Obtained data was evaluated by Student's t-test.

3-3-6. *Confocal laser scanning microscopy*

PECs stimulated with CM-chitin and F-Bs (described in 3-3) were placed on 8-well culture slide (BD Biosciences, USA) and incubated for 2 h to adhere phagocytes. The cells were stained with CellMask™ Orange (CMO, 5 □g/ml at final concentration, Invitrogen, Japan) fixed with formaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI, 10 μg/ml at final concentration, Dojin Chemical, Japan). The stained cells were covered with mounting reagent and analyzed by CLSM (A1si-90i microscope, Nikon, Japan).

3-3-7. *Bactericidal activity of phagocyte*

J774A.1 cells suspended in FBS/PS-free RPMI1640 were placed onto 12-well plate at 10^6 cells per well. Cells were stimulated with 2 mg/ml CM-chitin (or PBS, TCP for comparison) for 4 and 24 h, and washed with PBS twice. The cells re-suspended in the medium again placed onto another 12-well plate. S aureus or E coli were added at 5 x 10^6 cfu/well and cultured for 2 h under the mammalian cell culture condition. The cells were washed carefully with PBS to remove bacteria attached to cell surface, and lysed with water to release intracellular bacteria. The bacteria were stained by 1.7 μM thiazole-orange (TO) and 43 μM propidium iodide (PI) for 10 min and analyzed by flow cytometer. PI^+/TO^+ particles and PI^-/TO^+ particles were considered as dead and live bacteria, respectively.

3-4. Results

3-4-1. *Induction of phagocytes recruitment by CM-chitin*

Mice were injected ip with CM-chitin or β-TCP, and then further injected with Fluoro-labeled particles (F-Bs, F-Sa, F-Ec) to obtain PECs engulfing the particles. In all cases, total number of PECs stimulated by CM-chitin was almst double compared to that by β-TCP. F-Sa and F-Ec injection further increased the number of PECs. Flow cytometory analysis displayed that the PECs were mainly composed of macrophages ($F4/80^{+}Gr1^{+}$) and neutrophils ($F4/80^{-}Gr1^{+}$) (Figure 1a). CM-chitin significantly enhanced neutrophil recruitment, especially in F-Sa or F-Ec co-injection (Figure 1b). CM-chitin also increased macrophage recruitment. Two types of labeled-particles, F-Sa and F-Ec amplified the number of macrophage and neutrophil at CM-chitin stimulating group because of their somatic antigen.

3-4-2. *Chemokine expression*

Since CM-chitin enhanced phagocyte-recruitment for peritoneal cavity, the effect on chemokine expression in CM-chitin stimulation was examined. Out of those chemokines, two particular chemokines, monocyte/macrophage inducer MCP1 (32) and neutrophil inducer KC (33), showed remarkable expressions (Figure 2). CM-chitin stimulation of PECs increased level of MCP1 up to 25 fold as opposed to PBS stimulation, while β-TCP stimulation of PECs showed constant level up to 10 fold as opposed to PBS. Significantly high expression of KC was observed in CM-chitin stimulation at 1 h and there was no another remarkable expression at all time causes.

3-4-3. *Activation of phagocytosis by CM-chitin*

The CLSM images of PECs from mice co-injected with F-Bs were shown in Figure 4. PECs stimulated with CM-chitin engulfed F-Bs and the number of F-Bs engulfing cells was likely to higher than that of cells stimulated with β-TCP. Thus, the ratio of particle-engulfing cells was analyzed by flow cytometer. CM-chitin significantly enhanced engulfment of F-Bs by macrophages compared with β-TCP (Figure 3a). Similarly, CM-chitin increased phagocytosis of F-Sa and F-Ec by macrophages (Figure 3b). Phagocytosis of neutrophils was also increased by CM-chitin for F-Sa and

F-Ec (Figure 3b).

3-4-4. *RNS and ROS induction in macrophage*

RNS and ROS released from phagocytes were determined to estimate bactericidal activity in macrophage. CM-chitin treatment strongly increased the production of RNS in J774A1 and THP1, especially in J774A1 at 24 h after stimulation, and slightly enhanced ROS (Figure 5a). Co-stimulation of F-Bs scarcely affects the production of RNS or ROS. Base on the results of significant increase of RNS, RNA expression of iNOS was determined in J774A1. As shown in Figure 5b, CM-chitin specifically increased iNOS expression at 4 h after stimulation of J774A1.

3-4-5. *Bactericidal activity of macrophage*

In paper disk test (6 mm size paper-disc, Toyo Roshi Kaisha Ltd., Japan), 0.1, 1, 10 mg/ml CM-chitin did not inhibit bacterial growth of *E. coli* and *S. aureus*, and this showed that CM-chitin exhibited neither bactericidal nor bacteriostatic activity (data not shown). In order to ensure the contribution of CM-chitin to bactericidal activity, viability of intracellular bacteria was determined by flow cytometry. After co-incubation of bacteria with J774A.1, mortality rate of bacteria was evaluated by PI-intensity of killed-bacteria within TO-positive region (Figure 6a). The results indicated that mortality rate was significantly increased in the CM-chitin treated group (Figure 6b). The increase of mortality rate for *E. coli* was similar to that for *S. aureus*. Additionally, increase of cell-stimulation time showed no remarkable difference for both *E. coli* and *S. aureus*.

3-5. Discussion

We previously reported that CM-chitin induced the regeneration of hyaline cartilage in rabbits *in vivo* (29). In the operative treatment, any infectious disease was not observed in joint of those rabbits (personal communication). Additionally, our previous data indicated that CM-chitin stimulates phagocytes and induces several inflammatory cytokines and growth factor (30). These results prompted us to investigate whether CM-chitin contributes to enhance bactericidal activity of phagocytes and contributes to suppress bacterial infection.

In this study, we firstly demonstrated that CM-chitin induced a number of intraperitoneal phagocytes in mouse. The chemotaxis activity of CM-chitin corresponded to former experiment (30) and other reports for chitin or its derivatives (19-22, 34). The results of CLSM and flow cytometry analysis indicated that CM-chitin stimulation significantly increased phagocytosis activity of $F4/80^{+}Gr1^{+}$ macrophage and/or neutrophil (Figure 3,4). We particularly focused on the activity of macrophage, which is involved in both innate and acquired immune responses (35-37). Activated macrophage is associated with the production of large amounts of nitric oxide (RNS), superoxide (ROS), proinflammatory cytokines and involved in cytotoxicity and killing of microorganisms (38,39). Characteristic RNS production and iNOS expression of J774A.1 was remarkably high when J774A.1 was stimulated with CM-chitin (Figure 5). The results were consistent with the nitric oxide production of chitin-stimulated macrophage (34). Bactericidal activity test, which was evaluated by TO/PI staining of intracellular *E coli* or *S aureus*, indicated that both mortality rates of bacteria were increased in CM-chitin treated group (Figure 6). This indicated that antimicrobial activation by CM-chitin would be applicable for both gram positive and negative bacteria. We suggested in this study that CM-chitin augmented bactericidal activity through the increase of phagocytes and the enhancement of their phagocytosis activity and RNS production.

Previous reports for immune responses to chitin or its derivatives covered a broad range of topics. These researches showed that chitin derived inflammatory activation of macrophage resulted in production of inflammatory cytokines or several toxic intermediates such as RNS/ROS (5,20,34,40,41). In contrast, other researches showed that chitin derived

activation of alternative macrophage *in vivo* and resulted in production of chitinase-like proteins such as acidic mammalian chitinase (AMCase), arginase-1 or Ym1/Ym2 (1,21,22,42). Looking at the chitin-derived products in those studies, former researches suggested that chitin induced M1 (classical) macrophage involved in anti-bacterial activity, while latter researches suggested that chitin induced M2 (alternative) macrophage involved in allergic activity (43,44). Various factors, such as difference of ingredient, model animal or tested organ, would be considered to affect process of macrophage differentiation. However, one of the most important factors would be length of chitin. Muzzarelli et all. and Lee et al. suggested that chitin was dissolved by lysozyme or AMCase secreted from inflamed tissue, and then the disrupted chitin stimulated macrophage in a size-dependent manner (20,40). Lee et al. also gave a suggestion that intermediated-sized (40-70 μm) chitin activated M1-type macrophage (produce inflammatory cytokines), while small-sized chitin (< 40 μm) activated M2-type macrophage (produce anti-inflammatory cytokines) (20). Our previous result, in which CM-chitin firstly induced inflammatory cytokines (M1-type) and secondly induced TGF-β1 (M2-type), corresponds to the hypothesis (30). Furthermore, previous study showed that O-carboxymethyl group in CM-chitin enhanced the lysozyme-sensitivity and hydrolysis-efficiency of chitin (45,46). In this view, we hypothesized that CM-chitin was dissolved soon after i.p. administration into mouse and activated bacteriddal activity of M1-type macrophage. Following the activation of M1-type macrophage, lesser dissolved CM-chitin induced M2-type macrophage and resulted in production of TGF-β1.

In this study, we showed that CM-chitin enhanced the bactericidal activity of macrophage and it was suggested that the activity resulted from induction of M1-type macrophage in early-response. However, detailed mechanism for the interaction between CM-chitin and macrophage is still unknown. Further investigation will be needed to elucidate the mechanism.

3-6. Figures and figure captions

Figure 3-1. Phagocyte induction in peritoneal cavity by CM-chitin. PECs were collected at 24 h after CM-chitin injection, and followed by cell counting and flow cytometry analysis. a) Typical example of cell classification. Collected PECs were stained by anti-F4/40 antibody and anti-Gr1 antibody and the plotting charts were used to distinguish macrophage ($F4/40^{+}Gr1^{+}$) and neutrophil ($F4/80^{-}Gr1^{-}$). b) Cell number of phagocytes. Total number of PECs counted by hemocytometer was multiplied by the percentage of $F4/80^{+}Gr1^{+}$ cells or $F4/80^{-}Gr1^{+}$ cells. Each value is the mean ± SE of triplets. * and ** indicate the significances at $p<0.05$ and $p<0.01$, respectively, compared with β-TCP stimulation group.

Figure 3-2. Chemokine expression of PECs stimulated with CM-chitin. PBS, β-TCP and CM-chitin was administered into peritoneal cavity of mice. After 1, 4 and 8 h, PECs were collected and mRNA expressions of chemokines were measured by Q-PCR. Data are expressed as the mean ± SE of 3 independent experiments. mRNA expression was normalized to that of GAPDH and expression in PBS treated group was used as the standard. *, ** and *** indicate the significances at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with β-TCP stimulation group.

Figure 3-3. Flow cytometry analysis of phagocytosis enhancement by CM-chitin. a) Typical example of phagocytosis. After 24 h stimulation of PECs, FITC-labeled particles (F-Bs, F-Sa, F-Ec) were applied to PECs and followed by 4 h incubation. The incubated PECs were classified by F4/80, Gr1 labeling and then the distinguished macrophages were used to determine phagocytosis level. Fluorescent intensity indicates the phagocytosis level of each group. b) Mean fluorescent intensity (MFI) of each macrophage group. Each value is the mean ± SE of triplets. *, ** and *** indicate the significances at $p<0.05$, $p<0.01$ and $p<0.005$, respectively, compared with β-TCP stimulation group.

Figure 3-4. Microscopic observation of phagocytosis enhancement by CM-chitin. PECs were stimulated with CM-chitin or β-TCP for 24 h and followed by administration of FITC-labeled F-Bs. After 4 h, PECs were

collected, fixed and stained by DAPI and CMO. Prepared samples were observed by CLSM. F-Bs engulfed by phagocytes showed white color, while un-engulfed F-Bs showed green color.

Figure 3-5. Induction of RNS from macrophage cell line. a) Production of reactive nitrogen species (RNS) and reactive oxygen species (ROS). J774A.1 and THP1 were stimulated with PBS, β-TCP and CM-chitin for 24 h and RNS, ROS levels were measured by Griess and WST-1 methods, respectively. Bs showed the treatment group that was cultured with latex-beads. *, ** and *** indicate the significances at $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with β-TCP stimulation group. b) mRNA expression of inducible nitric oxide synthase (iNOS). J774A.1 was stimulated with PBS, TCP and CM-chitin and mRNA was extracted from J774A.1 at regular time intervals. mRNA expression of iNOS was measured by Q-PCR. Data are expressed as the mean ± SE of triplets. mRNA expression was normalized to that of GAPDH and expression in PBS treated group was used as the standard. *** indicates the significances at $p<0.005$ compared with β-TCP stimulation group.

Figure 3-6. Increase of bactericidal activity by CM-chitin. J774A.1 cell was precedently stimulated with PBS, β-TCP, CM-chitin and co-incubated with *S aureus* or *E coli*. After 4 or 24 h of co-incubation, J774A.1 cell was disrupted and intracellular bacteria were stained by propidium iodide (PI) and thiazole orange (TO) and followed by flow cytometry analysis. a) Typical example of bacteria plotting. Bacteria above the threshold of TO were used for determination of mortality rate. Mortality rate was evaluated by PI-intensity of TO+ bacteria. b) Mortality rate of S aureus or E coli. Each value is the mean ± SE of triplets. ** and *** indicate the significances at $p<0.01$ and $p<0.005$, respectively, compared with PBS stimulation group.

Figure 3-1

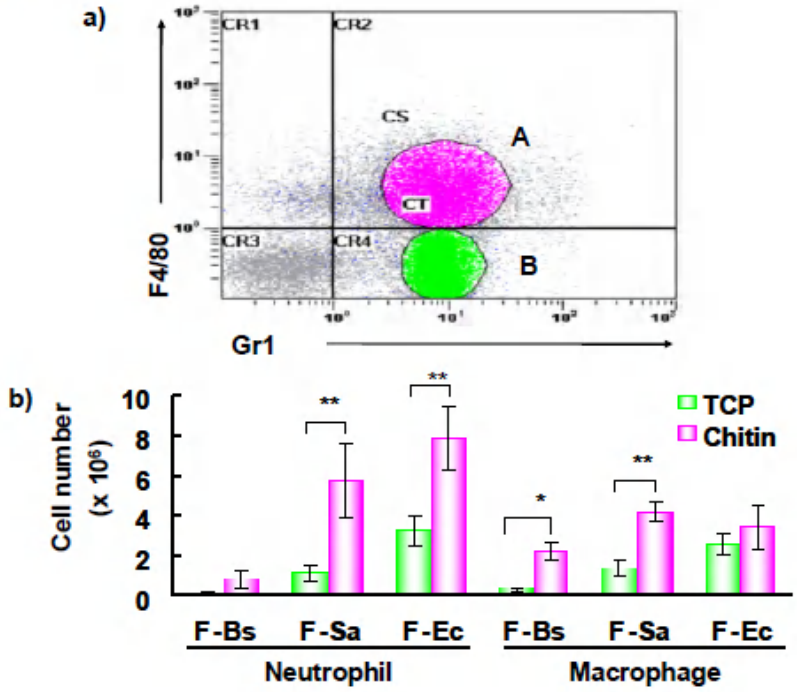


Figure 3-2

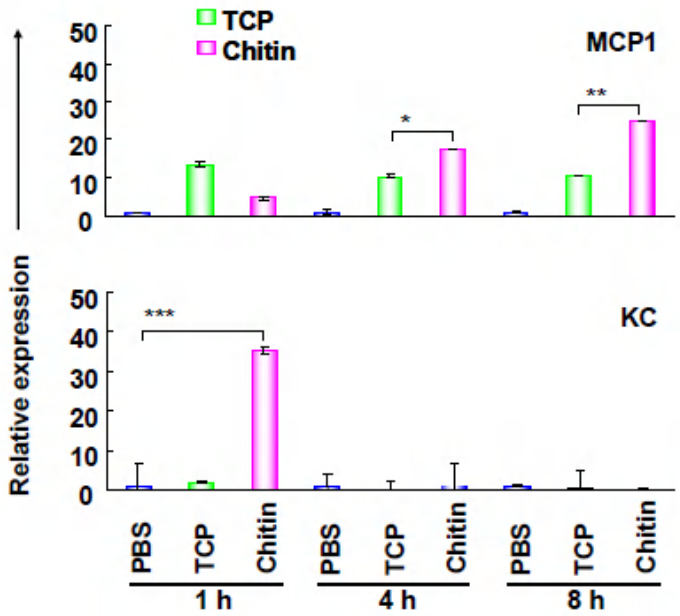


Figure 3-3

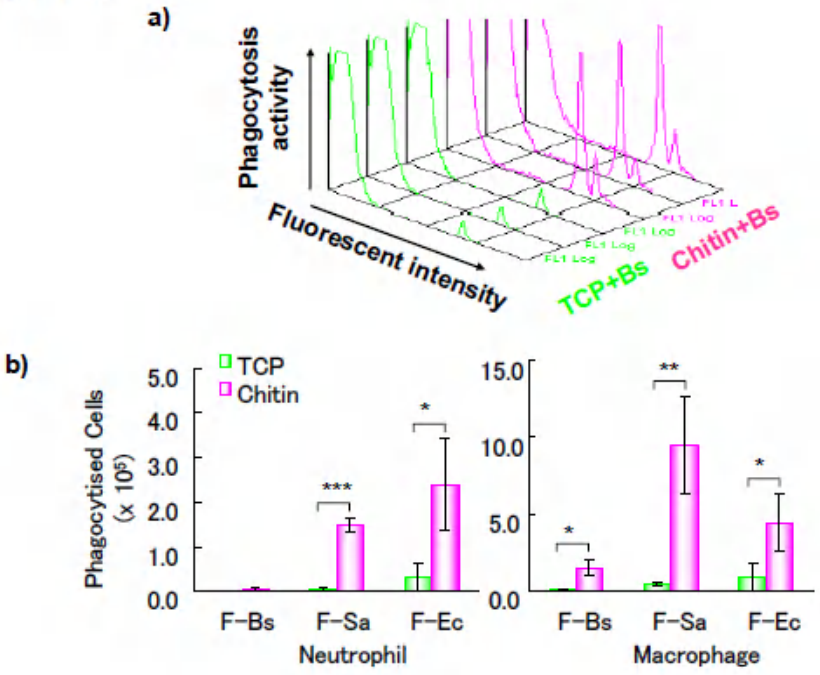


Figure 3-4

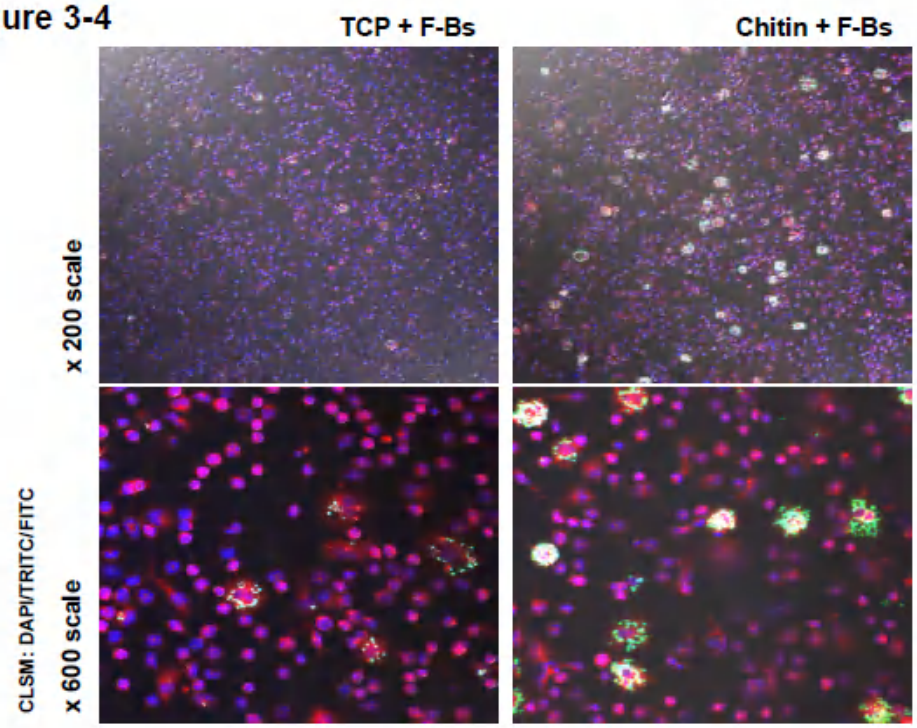


Figure 3-5

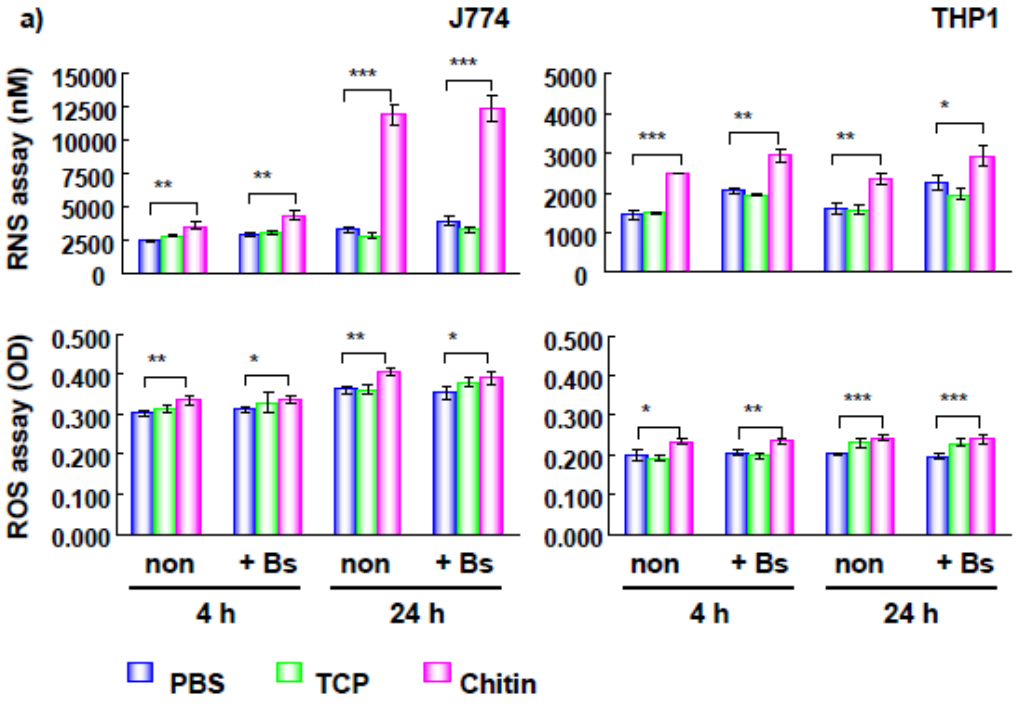


Figure 3-5

b)

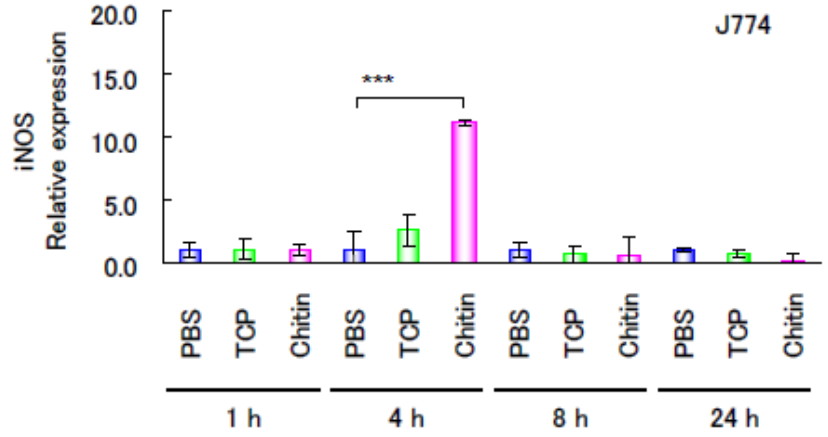
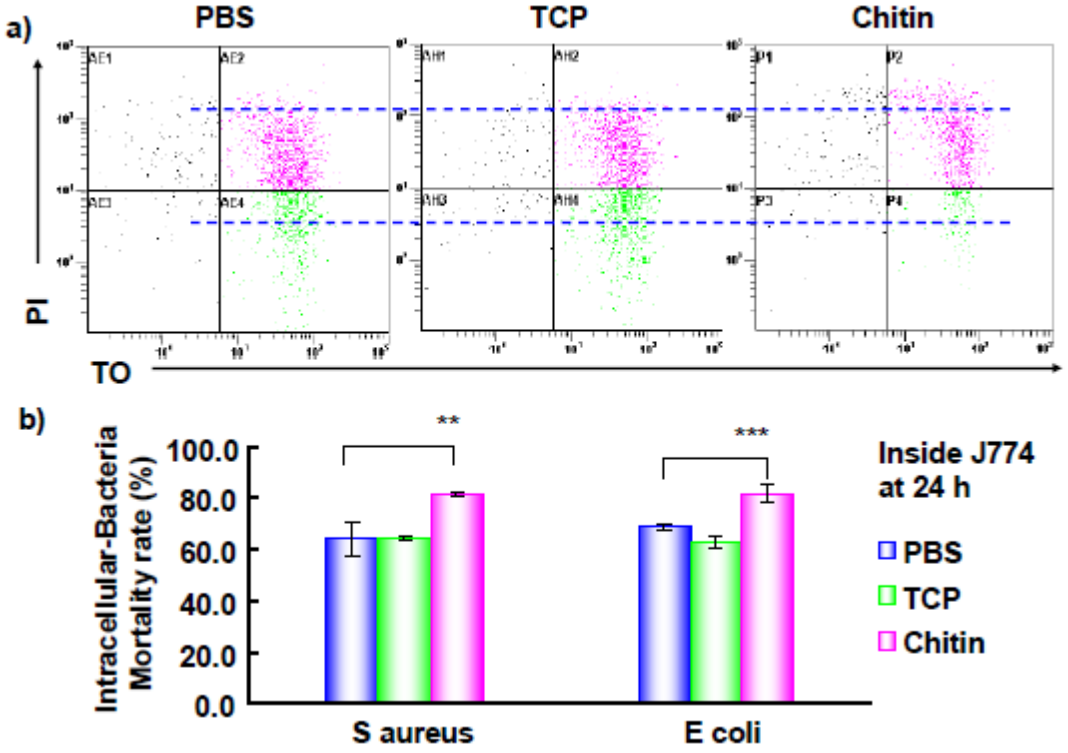


Figure 3-6


a)
PBS
TCP
Chitin
PI
TO
b)
Intracellular-Bacteria Mortality rate (%)
100.0
80.0
60.0
40.0
20.0
0.0
**
***
S aureus
E coli
Inside J774 at 24 h
PBS
TCP
Chitin

3-7. References

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Section 4

Conclusion

In the present study, we clarified three instances as mentioned below. First, carboxymethyl-chitin (CM-chitin) stimulated peritoneal exudate cells (PECs) and induced several inflammatory cytokines and one growth factor, TGF-β1. Second, culture supernatant of PECs stimulated with CM-chitin promoted *in vitro* chondrogenesis in micromass culture, and therefore CM-chitin would be a potent inducer of chondrogenesis via the induction of TGF-β1 in immune cells. Third, CM-chitin augmented bactericidal activity through the increase of phagocytes and the enhancement of their phagocytosis activity and RNS production. In conclusion, physiological activity of CM-chitin is due to the stimulating function for phagocytes, particularly macrophage, and the specific activation of phagocytes would be conductive to *in vivo* / *in vitro* chondrogenesis and bactericidal activity. The results achieved in this study will contribute to regeneration research and immunological research in near future. To elucidate the mechanism of activation, further investigation will be needed.