The establishment of a porcine rheumatoid arthritis model: Collagen induced arthritis minipig model

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1. Introduction

Rheumatoid arthritis (RA) is a common autoimmune disease that affects about 0.5–1% of the adult population in the world (1). RA is characterized by synovial hyperplasia, inflammatory cell infiltration, cartilage degradation, bone erosion, joint destruction and an increase in the levels of pro-inflammatory cytokines. Understanding of the disease pathogenesis and etiology, however, is as-yet insufficient for the development of new therapeutic strategies (2).

To explore novel therapeutic strategy, and in a bid to understand the pathogenesis of RA, several animal models have been established in the preceding decades; these include mouse, rat, guinea pig, and non-human primate (NHP) models. Of particular note are the rodent models, which have had induced arthritis via specific antigens as well as chemical reagents. These include models based on collagen induced arthritis (CIA), streptococcal cell wall arthritis, adjuvant induced arthritis, proteoglycan-induced arthritis, and serum transfer induced arthritis. In addition to the above, genetically modified TNF-α transgenic mice and K/BxN transgenic mice were developed as animal models for spontaneous RA (3).

Particularly, the preclinical research of RA has been enormously relied on CIA rodent models (4). Since it was first described by Trentham et al. in 1977, immunization to heterologous type II collagen (CII) has been widely applied due to the high incidence of arthritis with similar pathology of human RA (5,6); the histopathological changes associated with T-cell specific cellular and humoral immunity against CII were revealed in the CIA model, with moral immunity against CII were revealed in the CIA model, with some potential suitability of this test system as a large animal model for RA has been demonstrated.

With confirmation of the susceptibility to heterogeneous CII for arthritis induction in minipig, the potential suitability of this test system as a large animal model for RA has been demonstrated.

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rodents. Similarly, the translational potential is limited by innate differences in the anatomy, physiology and genetics between rodents and humans. Therefore, large animal model has become of main importance in the preclinical and pharmacological evaluations, but a large animal CIA model has, to date, not been successfully established (8,11).

Porcine disease models, owing to a greater similarity in the anatomy and physiology with their human equivalents, have proven to be more effective in aiding the understanding of human disease pathogenesis as well as in the development of new therapeutic substances. In light of this, the present study focused on establishing a large animal RA model by way of CIA minipigs. Assessments were made of the clinical, hematological, radiological and histopathological features of minipigs post immunization to heterologous CII. Collectively, CIA porcine model is expected to have a greater translational potential than the current rodent models, which enables researchers to study a wide range of long term trials or the clinical treatments requiring human-equivalent size models.

2. Methods

2.1. Ethics

All procedures, including induction of arthritis, animal care and animal termination, were approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Induction of arthritis

Arthritis was induced according to the previous DBA/1 CIA mouse protocol with minor modifications (6,12). A total of 11 specific pathogen free (SPF) minipigs (PWG Genetics, Singapore), 3–4 month old males with approximately 7 kg body weight, was employed in this study. They were divided into 3 experimental groups; the intradermal injection group (ID group) had 5 animals assigned, the intra-articular injection group (IA group) drew 3 animals, and the final 3 animals were placed in control group. All animals were sedated with an intramuscular injection of ketamine (10 mg/kg) and xylazine (2.5 mg/kg), and anesthesia was maintained by isoflurane inhalation. The ID group was administered with a mixture of 1 mg/ml/kg bovine type II collagen (CII; Chondrex, WA, USA) emulsified with an equivalent volume of complete Freund’s adjuvant (CFA; Chondrex) intradermally on Day 1. To maintain the stability of intradermal injections and to reduce the risk of possible skin ulceration, each 0.1–0.2 ml of mixture was distributed over 40 to 80 spots across the dorsal region. The second immunization was performed on Day 22 using same procedure except the use of incomplete Freund’s adjuvant (IFA; Chondrex) instead of CFA. The IA group received 1 mg/ml mixture of CII emulsified with equivalent volume of CFA or IFA on Day 1 or Day 22, respectively, via direct injection into both the knee joint cavities. Control group received injections of an equivalent volume of phosphate buffered saline (PBS; Sigma, MO, USA) via both the ID and IA routes.

2.3. Gross observation and clinical evaluation

The clinical evaluations and body temperatures were recorded every three days from the second immunization day (Day 22) to Day 43 by three separate personnel. The criteria of the clinical evaluation at each limb were based on previously established standards, and were summarized in Table 1 (6). Rectal temperatures were measured using a digital centigrade thermometer. Hind paw thickness was measured in triplicate by three different evaluators with digital caliper ruler at the widest region of the tarsal joint on Day 22 and Day 43. The difference of hind paw thickness was calculated by subtracting Day 22 values from those on Day 43.

2.4. Radiological and hematological assessment

The tarsal joints of all groups were scanned for the morphological changes on Days 22 and 43 using a C-arm X-ray (Zen-2090, Genoray, Sungnam, Korea). Upon sacrifice on Day 43, the tarsal joints were harvested and subsequently scanned by microtomography (micro-CT; SMX-100CT, Shimadzu, Kyoto, Japan) to examine structural changes on the tarsal joints in response to arthritis development. Additionally, complete blood counts (CBC) were analyzed using an ADVIA 2120 Hematometry Analyzer (Siemens, NJ, USA) on Days 1, 15, 29 and 43; special attention was paid to the total number of WBCs, neutrophils, lymphocytes and monocytes in a bid to monitor any inflammatory response.

2.5. Histopathological assessment

All groups were euthanized on Day 43 with a thiopental sodium overdose. The metatarsophalangeal joints were harvested and fixed in 10% formaldehyde (Sigma) for 3 days. Fixed samples were then trimmed in 2 cm width × 2 cm length × 2 mm thickness, decalcified with Decalifying Solution-Lite (Sigma) for 24 h, dehydrated and embedded in paraffin. Slides of 4 μm sections were stained with Masson’s trichrome (Sigma) as well as hematoxylin and eosin (H&E; Sigma) before being observed under a light microscope (Nikon, Tokyo, Japan). The thickness of randomly selected 5 non-calcified cartilage regions was measured using the NIS elements program (Nikon).

2.6. Statistical analysis

One-way ANOVA was conducted in SPSS (IL, USA) with a Games-Howell post-hoc test to discern significant differences. Data were presented as the mean ± standard error of the mean (SEM). The p value less than 0.05 was considered as significant difference.

3. Results

3.1. Gross observation

One minipig in the ID group perished shortly after the first immunization; it was speculated that the pig suffered accidental intravenous injection of CII emulsified with CFA, but no further examination was conducted. Another minipig from the same group showed no clinical changes up to Day 43 and was therefore considered an unsuccessful induction. Symptoms of swollen joint such as the disappearance of plantar creases, thickened metatarsal joints and increased distance of each digit were featured prominently in the other 3 animals in the ID group, as well as all 3 animals in the IA group. Cases representative of the aforementioned symptoms may be observed in Fig. 1.

3.2. Clinical assessment

The clinical score of the ID group was significantly higher (p < 0.05) than the IA group from Day 28 onwards (Fig. 2a). The maximum clinical score was reached into 1.95 ± 0.3 or 1.25 ± 0.19 points in the ID or IA group at terminal end point, respectively. Limb swelling and erythema in both immunized groups were limited to distal regions such as the metacarpal/metatarsal joints, digits and palms, as opposed to more severe clinical signs including severe
**Table 1**
The criteria of the clinical evaluation.

<table>
<thead>
<tr>
<th>Severity score</th>
<th>Degree of inflammation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No evidence of erythema and swelling</td>
</tr>
<tr>
<td>1</td>
<td>Erythema and mild swelling to the carpals/tarsals or ankle/knee joint</td>
</tr>
<tr>
<td>2</td>
<td>Erythema and mild swelling extending from the carpals/tarsals to ankle/knee</td>
</tr>
<tr>
<td>3</td>
<td>Erythema and moderate swelling extending from the carpals/tarsals to ankle/knee</td>
</tr>
<tr>
<td>4</td>
<td>Erythema and severe swelling across the ankle/knee, arm/foot and digits</td>
</tr>
</tbody>
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**Fig. 1.** Gross observation of hind legs on Day 22 and Day 43. Both immunized groups showed the swollen metatarsal joints and palms on Day 43 in comparison with those of Day 22. Red arrows represented the morphological changes in regards to thickened joints, broadened inter-digital distance and disappearance of the plantar creases due to swelling. On the other hand, there was no evidence of the incidence of arthritis in control group between Day 22 and Day 43.

**Fig. 2.** Clinical score, hind paw thickness and body temperature. (a) Each limb of animals was scored separately according to the standards for the clinical evaluation every three days, and the average of points was calculated. (b) The difference of hind paw thickness was calculated by subtracting Day 22 values from those on Day 43. (c) Body temperature from rectum was measured every three days. X axis indicated days of study from Day 22 to Day 43, and Y axis presented values of each evaluation. *p < 0.05 vs. control, #p < 0.05 vs. the IA group.
erythema, ankylosis and extended swelling across the whole limb (Fig. 1). In comparison with control group, the ID group presented significant ($p < 0.05$) increase of hind paw thickness on Day 43 (Fig. 2b) as well as a significantly ($p < 0.05$) higher body temperature from Day 25 to Day 31 (Fig. 2c). However, no significant difference in the aforementioned was observed between the IA and control group.

3.3. Radiological examination

The metatarsal/tarsal joints of both immunized groups were examined with a C-arm X-ray, and findings included that indistinct condyle surface and blurred margin of the joint were not identified at joints in between immunized and control group on Day 22, but progressively developed and appeared in immunized groups on Day 43 (Fig. 3a). Harvested tarsal joints under images on micro-CT showed clear arthritic manifestations including joint deformities and dislocations when samples from both immunized groups were compared with those of control group (Fig. 3b). The ID group, in particular, presented more obvious morphological changes in the joint than the IA group.

3.4. Hematological assessment

Both immunized groups recorded a significantly ($p < 0.05$) increased WBC count 2 weeks after the first immunization, and that of the ID group showed the highest peak 1 week after the second immunization (Fig. 4a). Before animal termination on Day 43, the number of WBC in both immunized groups was sustained as same level as control group. The number of neutrophil, however, remained elevated significantly ($p < 0.05$) in both immunized groups during whole experimental period than control group (Fig. 4b). Furthermore, the ID group recorded a significantly ($p < 0.05$) higher level of lymphocytes 1 week after the second immunization (Fig. 4c). While the total number of monocytes was elevated in both immunized groups, it was not significantly important (Fig. 4d).

3.5. Histopathological assessment

The histopathological assessments of the metatarsophalangeal joint were conducted. Randomly selected 5 non-calciﬁed cartilage regions showed signiﬁcantly ($p < 0.05$) degraded thickness in the ID group in comparison with the IA and control group (Fig. 5a–c). However, destructed cartilages could be seen in both immunized groups (Fig. 5h and j). Angiogenesis as well as increased inﬁltration of mononuclear cells was observed extensively in the subchondral region adjacent to the damaged articulation site in both immunized groups, while most of mononuclear cells were only exhibited within the bone marrow in control group (Fig. 5d–f). In addition, the clusters of the inﬁltrated mononuclear cells in the subchondral region were revealed in both immunized groups (Fig. 5i and k).

Fig. 3. Radiological assessment by C-arm X-ray and the examination of the tarsal joints by micro-CT. (a) Indistinct condyle surface and blurred margin of the joint were not identiﬁed at joints between immunized and control groups on Day 22, but these ﬁndings were progressively developed and appeared in merely immunized groups on Day 43. These clinical ﬁndings on Day 43 were indicated as red arrows. (b) Harvested joints in both immunized groups on Day 43 were compared with control group, and examined for their deformities by micro-CT. Marks of asterisks were placed above the regions where deformities and dislocation could be observed in both immunized groups.
4. Discussion

The preceding decades have seen the establishment of both induced and genetically modified spontaneous models of arthritis (3,11,13). CIA, in particular, has been widely used due to its well-characterized mechanism of arthritis induction through the administration of heterogeneous CII (13,14). This autoimmune response against heterogeneous CII is led by the T helper cells, which play a role in the adaptive immune system in combination with pro-/anti-inflammatory cytokines and/or their receptors. Since CII is rich protein in the articular cartilage, the cartilage begins to be destroyed by the production of CII-specific antibodies as well as cellular reactivity to CII (15,16). Due to similarities in the clinical presentation, histopathology and immune response between CIA animal and human RA, novel therapeutic strategies have frequently been subjected to investigations utilizing CIA in mice, rats, guinea pigs and NHP (8,14,17,18).

Despite the wealth of information on rodents as a base species for CIA, RA studies involving rodents may not translate well to humans due to innate differences in the size, physiology and lifespan. Minipig-based models, with a higher level of similarity to their human equivalents in terms of the physical, hematological, histopathological and etiological properties, were tested for susceptibility to arthritis by the administration of heterogeneous CIA. This commonly used CIA model has been subjected to investigations utilizing CIA in mice, rats, guinea pigs and NHP (8,14,17,18).

In the present study, it was found that while minipigs showed the onset of arthritis, the arthritis did not progress aggressively at the clinical observations. The ID group had a clinical score of 1.95 ± 0.3 points, only presenting with swelling in distal regions such as the metacarpal/metatarsal joints, digits and palms (Fig. 2a). Symptoms of a level of arthritis severity such as severe erythema, ankylosis and extended swelling across the entire limb, able to be scored as 3–4 points, were not obvious during the observation period. DBA/1 CIA mouse model, generally presenting with a high level of severity post immunization, has also shown varied incidence results evaluated as 2–4 points (6,12,19–21). Similarly, CIA models using Balb/c mice scored between 2 and 3 points post immunization with CII (22,23). Further, increased thickness in hind paw is considered as one of the main physical changes associated with the onset of arthritis in CIA animal (12,19). Upon observation, the ID group presented the tarsal joint swelling as well as a significant (p < 0.05) elevation in thickness of the hind paw (2.12 mm ± 0.42 mm) over control group (0.42 mm ± 0.3 mm). IA group presented results midway in-between the ID and control group (Fig. 2b).
While humans suffering from chronic RA commonly present with anomalous hematology such as anemia, neutropenia and thrombocytopenia, these symptoms are generally not fully observed in CIA animal models due to the nature of arthritis induced by heterogeneous CII; the exogenous inducer mainly causes acute arthritis with an early inflammatory response, instead of the chronic arthritis prevalent in humans (8,24,25). CIA NHP model may present with two different types of arthritis, namely of the early and late-onset type. The latter type did not show neutropenia even after 126 days of observation (26). In Fig. 4, both immunized groups showed elevations of the total number of WBC and neutrophil during days between the first and second immunization. Such inflammatory response post the first immunization could be supported by common observation during CIA protocol; the intradermal immunization to mice with CII emulsified with CFA gave rise to an inflammation at the injection site for 1–2 weeks after the first immunization (6). The first signs of arthritis development, away from injection site, are visible between Days 18 and 25 after the first immunization in most of the CIA mice (2,6,21). In the present study, the inflammatory reaction at the injection site was maintained for 3 weeks, therefore, it was speculated that this reaction resulted in the hematological changes of the total number of WBC and neutrophil at 2 weeks after the first immunization (Fig. 4a and b). It was hard to observe any incidence of arthritis such as swelling in the carpal/tarsal joints during days between the first and second immunization, but mild swelling at those joints started to be seen from Day 25 (Fig. 2a). Furthermore, both immunized groups showed significantly ($p < 0.05$) elevated total WBC and neutrophil counts as well as increased tendency of lymphocyte and monocyte counts at Day 29 in comparison with those levels of control group. These changes have been taken as an indication of a systemic response against exogenous CII post immunization.

WBC infiltration into the cartilage has been linked with cartilage degradation and bone erosion in CIA animals (2,12). Both immunized groups presented degraded and destructed cartilage (Fig. 5a–c, g, h and j), as well as an increase in the number of infiltrated mononuclear cells into the subchondral region over control group (Fig. 5d–f, i and k). Angiogenesis across the subchondral region, a compensatory mechanism in response to hypoxia during synovial expansion, has been regarded as an important observation in human RA (27). In the present study, both immunized groups displayed increased angiogenesis with red blood cell filled vessels near the subchondral region, marking another point of similarity between these groups and human RA.

The radiological features of RA have also been well documented, especially with regards to synovitis, effusion, osteoporosis, narrowing of the joint space and bone erosion (28). The radiological examination using a C-arm X-ray revealed no obvious difference of joints between immunized and control groups on Day 22, therefore, it was speculated that there was no onset of arthritis till Day 22 in both immunized groups. But it was clearly identified that narrowing of the joint space, and indistinct condyle surface and blurred margin of joints were observed in immunized groups on Day 43 in comparison with those on Day 22 and control group (Fig. 3b). Additionally, micro-CT scans showed irregular grooves at the bone margins, as well as a receded or nonexistent intra-articular joint.

Fig. 5. Histological assessment in the metatarsophalangeal joints for the measurement of the cartilage thickness, cartilage destruction and mononuclear cell infiltration. (a–c) The cartilage from control, the ID and IA groups was stained as blue by Masson’s trichrome staining. (g) Randomly selected 5 non-calcified cartilage regions in Masson’s trichrome staining were measured about those thicknesses. The cartilage thickness was presented as mean ± SEM. * $p < 0.05$ vs. control, # $p < 0.05$ vs. the IA group. (h, j) Cartilage destruction could be observed in both immunized groups. (d–f) H&E staining was employed to determine the infiltration of mononuclear cells. Angiogenesis of small vessels was marked as black arrows. (i, k) Blank arrows indicated the cluster of the infiltrated mononuclear cells in both immunized groups. Magnifications are ×40 (a–f) and ×200 (h–k), respectively.
space and dislocations of the tarsal joints in both immunized groups over control group (Fig. 3b). The aforementioned changes were observed at a greater degree of severity in the ID group over the IA group.

A variety of cytokines are related with the pathogenesis of CIA rodent model. Ankle joint extracts in CIA rodent model present drastic elevations of the inflammatory cytokines such as IL-1β, TNF-α, and IL-6 (29). While TNF-α and IL-2 are associated with T helper cell type 1 response to activate disease-booster, IL-4 is related with T helper cell type 2 to inhibit the arthritis (14). The immunosuppressive properties of stem cells in CIA rodent model are characterized by association with increased levels of IL-10 as the anti-inflammatory cytokine as well as decreased levels of IL-1 as the pro-inflammatory cytokine (30). Unfortunately, although the present study and other studies that described large animal CIA model clearly provided the evidence of the onset of arthritis, a limitation was the lack of further study regarding changes of cytokine profile (18,26).

In the present study, the susceptibility of minipigs to arthritis induction via administration of heterogeneous CII emulsified in CFA and IFA was confirmed with showing symptoms associated with RA, such as joint swelling, increased joint thickness, the hematological alteration, degraded or destructed cartilage and infiltration of monocyte to the subchondral region. Furthermore, the changes in regards to narrowing of the joint space, indistinct condyle surfaces and the joint deformities were identified by the radiological observation. The aforementioned symptoms also generally appeared more severe in the ID group than the IA group. In light of the findings put forth in this paper, it is hoped that CIA minipig model may be recognized as a potentially suitable test system for further research.

Conflicts of interest

No conflict of interest.

Author’s contribution

WJL designed the project, performed collection and/or assembly of data and wrote manuscript. JYK conducted the animal experiment and evaluation of arthritis. TPW performed the histopathological process. LSP involved in the administrative support, provided overall guidance, monitored the project and revised present manuscript.

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References