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Review
Young Investigator Award

Development of outcome measures according to dystrophic phenotypes in canine X-linked muscular dystrophy in Japan

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Abstract: Duchenne muscular dystrophy (DMD) is an X-linked lethal muscle disorder characterized by primary muscle degeneration. Therapeutic strategies for DMD have been extensively explored, and some are in the stage of human clinical trials. Along with the development of new therapies, sensitive outcome measures are needed to monitor the effects of new treatments. Therefore, we investigated outcome measures such as biomarkers and motor function evaluation in a dystrophic model of beagle dogs, canine X-linked muscular dystrophy in Japan ($CXMD_J$). Osteopontin (OPN), a myogenic inflammatory cytokine, was explored as a potential biomarker in dystrophic dogs over the disease course. The serum OPN levels of $CXMD_J$ dystrophic dogs were elevated, even in the early disease phase, and this could be related to the presence of regenerating muscle fibers; as such, OPN would be a promising biomarker for muscle regeneration. Next, accelerometry, which is an efficient method to quantify performance in validated tasks, was used to evaluate motor function longitudinally in dystrophic dogs. We measured three-axis acceleration and angular velocity with wireless hybrid sensors during gait evaluations. Multiple parameters of acceleration and angular velocity showed notedly lower values in dystrophic dogs compared with wild-type dogs, even at the onset of muscle weakness. These parameters accordingly decreased with exacerbation of clinical manifestations along with the disease course. Multiple parameters also indicated gait abnormalities in dystrophic dogs, such as a waddling gait. These outcome measures could be applicable in clinical trials of patients with DMD or other muscle disorders.
Key words: accelerometry, canine X-linked muscular dystrophy in Japan ($CXMD_J$), Duchenne muscular dystrophy, osteopontin, outcome measure

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked muscle disorder characterized by primary muscle degeneration [1]. Its prevalence in the population is estimated to be 1 in 5,000 male newborns. A mutation in the *DMD* gene results in the absence of dystrophin, a structural protein in muscle fibers, which leads to weakened muscle fibers following contractive force [2]. The histologic features of DMD include muscle fiber degeneration with secondary cellular inflammation and ineffective muscle fiber regeneration, with the muscle eventually being replaced by fibrous and adipose tissue [3]. Patients with DMD manifest progressive muscle weakness and

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contracture [4, 5]. The early symptoms of DMD, which include gait disturbance, frequent falls, and difficulty climbing stairs, usually appear by 2–5 years of age, with loss of ambulation occurring between 9 and 12 years of age. Respiratory or cardiac failure causes death in the third decade.

Steroid therapy for muscle weakness is currently applied in the clinical setting to prolong muscle strength and ambulation; however, it is not a cure for DMD [5–7]. Various therapeutic strategies, such as pharmaceuticals and gene and cell therapy, have been proposed and explored in human trials [8–12]. Among these strategies, exon skipping and stop codon read-through, which are designed to restore dystrophin expression, are the most advanced [12]. A morpholino antisense oligonucleotide, NS-065/NCNP-01 (viltolarsen), which effectively induces exon 53 skipping in the *DMD* gene, was recently established to be efficacious and safe in human trials [13–15] and was approved in Japan and the USA in 2020. In the development of new therapeutic interventions, surrogate endpoints have become increasingly important to evaluate treatment effects in detail. Therefore, sensitive outcome measures, including biomarkers and motor function evaluations, are needed.

Overview of Canine Models for DMD

Golden retriever muscular dystrophy (GRMD)

To explore the efficiency of the different preclinical treatments for DMD, there is a need for animal models that show the dystrophic phenotypes. Spontaneous genetically homologous dystrophin-deficiency has been identified in several species, including mice and dogs. The X-linked muscular dystrophy (*mdx*) mouse is the most widely used animal model for DMD, even though its mild phenotype does not mimic the severe symptoms of human DMD [16, 17]. By contrast, canine dystrophic models share the severe myopathy and DMD conditions [18–20]. Novel animal models for DMD, such as rats [21, 22], rabbits [23], pigs [24], and rhesus monkeys [25], have been generated using genome editing methods and subsequently examined for their phenotypes [26].

Canine dystrophinopathies have been reported in a number of canine breeds [27–29]. Canine X-linked muscular dystrophy, which is compatible with degenerative myopathy, was documented in golden retrievers in the 1980s [18, 30–32] and recognized as a genetic homologue of DMD due to the lack of dystrophin protein [33]. GRMD has been established in a breeding colony of golden retrievers by reproducing dystrophic dogs according to an X-linked pattern of inheritance [28, 32, 34]. Dogs with GRMD are dystrophin deficient because of a splice-site mutation resulting from a single base change in the 3' consensus splice site of intron 6 [35]. Exon 7 is then skipped, which is predicted to result in a termination of the dystrophin reading frame within its N-terminal domain in exon 8.

In GRMD, the histologic features include widely observed segmental degeneration of muscle fibers (hyaline fibers, myophagocytosis) and regeneration (small basophilic fibers) [30, 31], following the selective involvement of muscles such as the diaphragm, tongue, and limb muscles in the neonatal phase [36, 37]. Progressive changes include the development of severe fiber size variation, endomysial and perimysial fibrosis, fat infiltration, and alterations in the fast and slow fiber-type patterns over the disease course [19, 38]. In clinical phenotypes, a severe, lethal, neonatal form of the disease has been recognized in which GRMD pups do not survive beyond 2 weeks of age [18, 28, 36, 37]; GRMD pups that survive are only mildly affected and tend to stabilize at around 2 weeks of age [28]. Clinical signs are commonly observed as an onset of generalized muscle weakness and posture and gait abnormalities with selective muscle atrophy and hypertrophy at between 6 and 9 weeks of age; these exacerbations markedly progress until 6 to 9 months of age [18, 28, 30, 39]. It has also been noted that the pelvic limbs appear stiff and are simultaneously advanced ("bunny hopping"), resulting in a stiff and shuffling (waddling-like) gait. Other clinical features include poor weight gain, kyphosis, splaying of the limbs, fatigue with exercise, neck stiffness, resistance to jaw opening, enlargement of the base of the tongue, pharyngeal and esophageal dysfunction (dysphagia and regurgitation), and occasional respiratory and cardiac distress.

Canine X-linked muscular dystrophy in Japan ($CXMD_J$)

$CXMD_J$ is a dystrophic model of beagle dogs established as a breeding colony by inseminating beagles with the sperm of GRMD dystrophic dogs at the National Center of Neurology and Psychiatry [40]. $CXMD_J$ dystrophic dogs have a mutation in the *DMD* gene analogue to GRMD and lack dystrophin in their muscle tissue [40–42]. The clinical and histologic features of $CXMD_J$ have been found to be similar to those observed in GRMD [20]. The clinical manifestations of $CXMD_J$ can be evaluated from clinical signs, such as gait and mobility disturbances, limb and temporal muscle atrophy, drooling, macroglossia, dysphagia, and abnormal sitting posture, by classifying the severity on a scale of 1 to 5 (from grade 1, none, to grade 5, severe) [20, 43]. The early clinical signs, including gait disturbance and distal

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limb and temporal muscle atrophy, are observed at around 2 months of age, corresponding to the onset of muscle weakness [20]. High mortality of $CXMD_J$ neonates has also been observed (e.g., a mortality of 32.3% by 3 days of age in the third generation) [20], confirming our results indicating that initial pulmonary respiration causes massive diaphragm injury followed by respiratory dysfunction [44].

Pathophysiological investigations in $CXMD_J$

Various studies on $CXMD_J$ have revealed the novel characteristics of dystrophic pathology. In the diaphragm of $CXMD_J$, the muscle fiber-type composition is found to switch from the fast type to predominantly the slow type over the disease course [45]. Matrix metalloproteinases (MMPs), which are proteolytic enzymes that degrade extracellular matrix components, are upregulated in the dystrophic muscles; MMP-9 and MMP-2 are related to degenerating muscle fibers with inflammatory cells and regenerating muscle fibers, respectively [46]. The cardiac phenotypes of $CXMD_J$ show prominent deep Q-waves and increased Q/R ratios in leads II, III, and aVF on electrocardiography by 6 to 7 months of age [47], and they show a decreased peak radial strain rate during early diastole in the posterior segment of the left ventricle on echocardiography after 8 months of age [48]. Selective vacuolar degeneration of Purkinje fibers is found as early as 4 months of age, suggesting an association between cardiac conduction abnormality and fatal arrhythmia [49]. Myocardial fibrosis in the left ventricular posterobasal wall has been observed in several dystrophic dogs by 21 months of age [47].

Preclinical investigations of therapies and outcome measures in $CXMD_J$

Preclinical studies of new therapies for DMD have been conducted in $CXMD_J$. Multi-exon skipping using morpholino antisense oligonucleotide cocktails, which induce exon 6 and 8 skipping in the *DMD* gene of $CXMD_J$, has been found to restore the dystrophin reading frame [42, 50–52]. The systemic injection of antisense drugs has been shown to induce therapeutic levels of dystrophin expression and improve the dystrophic phenotypes, including histology, clinical manifestations, motor dysfunction, and cardiac conduction abnormalities [42, 50, 53–55]. Recombinant adeno-associated virus (rAAV) vectors transduce the microdystrophin gene into muscle fibers and ameliorate the dystrophic phenotypes of $CXMD_J$ [56–60]. Although host immune responses result in low and transient expression of transgene products, the efficiencies have been improved in rAAV-treated $CXMD_J$ by co-administration of immunosuppressants [56], rAAV serotype selection [57, 60], immune tolerance [59], and injection of multipotent mesenchymal stromal cells (MSCs) [60]. The injection of MSCs, which are mesoderm-derived multipotent cells derived from bone marrow and dental pulp that have immune-modulating effects [61], results in the formation of myofiber-like tissue and downregulates severe inflammation in the dystrophic muscles of $CXMD_J$ [61–63].

Novel outcome measures have been concomitantly developed in $CXMD_J$. Noninvasive evaluation methods, such as magnetic resonance imaging (MRI) and serum biomarkers, are used to examine the resulting temporospatial pathological changes. Dystrophic muscle lesions can be precisely assessed using conventional MRI sequences; chemical shift selective T2-weighted imaging has been shown to be sensitive for detecting muscle necrosis and inflammation by selectively canceling fat signals [64]. MicroRNAs (miRNAs), noncoding small RNAs, are considered candidate serum biomarkers for DMD. Among miRNAs, miR-1, miR-133a, miR-188, and miR-206 are elevated in serum and muscle tissues of $CXMD_J$, and miR-188 and miR-206 have been found to play roles in muscle regeneration [65–67].

Osteopontin (OPN) as a Serum Biomarker in Dystrophic Pathology

Serum biomarkers for DMD

Clinical biomarkers are necessary for the diagnosis of the disease, classification of its severity, and evaluation of therapeutic effects. Serum creatine kinase (CK) is a primary biomarker for a sensitive diagnosis of DMD that reflects muscle damage [68, 69]. However, serum CK is not sufficient for the evaluation of therapeutic effects, because its levels decrease with the progression of dystrophic disease, which corresponds to the wasting away of skeletal muscle. New candidate serum biomarkers have been explored to develop an evaluation method for dystrophic conditions [70]. MMP-9 and its endogenous inhibitor, tissue inhibitor of metalloproteinase (TIMP)-1, are strongly suggested to be DMD biomarkers [71, 72]. Serum miRNAs such as miR-1, miR-133, and miR-206 have been shown to be elevated in human DMD, as observed in $CXMD_J$ [66, 73, 74]. The levels of various cytokines and growth factors in the blood have also been found to be elevated in association with dystrophic pathology, including tumor necrosis factor-α, interleukin (IL)-1, IL-6, IL-13, interferon-γ, transforming growth factor-β, and basic fibroblast growth factor [70, 75–79]. Serum levels of several proteins are correlated with the clinical severity in ambulatory and nonambulatory patients with DMD [71, 76, 80].

OPN in dystrophic pathology

OPN, a phosphorylated glycoprotein synthesized in various tissues and cells that are involved in inflammation, tissue remodeling, and tumorigenesis [81, 82], has been reported to be a potential new candidate for a DMD biomarker. As an immobilized matricellular protein and a soluble molecule with cytokine functions, the secreted form of OPN is capable of interacting with CD44 and various integrins to mediate cell–cell and cell–matrix interactions and activate intracellular signaling pathways [81, 83]. The effects of OPN binding to target cells, such as promoting cell attachment, proliferation, migration, and chemotaxis, are modulated through cleavage by thrombin, as well as MMP-3, MMP-7, and MMP-12 [84–86].

In injured or dystrophic muscles of rodent models, OPN is expressed in immune cells, myoblasts, and damaged or regenerating muscle fibers [87–90]. OPN deficiency in the muscles of *mdx* mice leads to reduced inflammatory cell infiltration and skewed M1/M2 macrophage polarization, as well as the subsequent alleviation of fibrosis via TGF-β signaling [88, 91, 92]. Therefore, OPN could be a therapeutic target as a pro-inflammatory and pro-fibrotic molecule. Meanwhile, OPN also plays important roles in aiding the fusion and differentiation of myoblasts, primarily during the early phases of myogenesis, leading to the promotion of muscle regeneration [83, 90, 93–95].

In patients with DMD, OPN is detected in mononuclear cell infiltrates, on some muscle fiber surfaces, in regenerating fibers, and in calcified fibers [96]. Among human OPN isoforms, including OPN-a, OPN-b, and OPN-c, OPN-a is especially abundant and functionally active in the skeletal muscle microenvironments [97]. In addition, OPN may influence the severity of disease in patients with DMD, because single-nucleotide polymorphism (SNP) in the *SPP1* (*OPN*) promoter (rs28357094) has been shown to be correlated with DMD severity in a number of clinical trials [98–100] and with muscle size in healthy females [101]. The rare G allele of rs28357094 reduces its transcriptional activity compared with the ancestral T allele [102] but, conversely, enhances mRNA expression responsive to glucocorticoid drugs and estrogen [100, 101, 103].

Investigations of OPN in $CXMD_J$

In $CXMD_J$ dystrophic dogs, we have previously found that OPN has been upregulated in dystrophic diaphragms before initial pulmonary respiration at birth, suggesting the participation of OPN in a dystrophic muscle environment, even in the early phase [44]. It is therefore expected that OPN could potentially be a new biomarker for DMD that is expressed in a unique manner. We then monitored and compared serum OPN levels in $CXMD_J$ dystrophic dogs over the disease course with the levels of other serum markers, including serum CK [43]. Blood samples of dystrophic and wild-type (WT) dogs were collected before birth by caesarean section, at 1 h after a birth, at 3 and 6 weeks of age, at 2, 3, 6, and 9 months of age, and at 1 and 2 years of age. Serum OPN levels in dystrophic dogs were elevated and revealed a completely different pattern compared with serum CK, an established primary biomarker for muscle injury. Serum OPN levels were significantly elevated in dystrophic dogs compared with those in WT dogs before birth, at 1 h after birth, and at 3 months of age; those at 3 weeks of age remained higher in dystrophic dogs, but not significantly higher, compared with those of the WT dogs. Meanwhile, serum CK levels were significantly higher in dystrophic than in WT dogs at all age points, and post-birth levels were substantially elevated compared with those before birth, thus confirming our observation that massive diaphragm injury is caused by initial pulmonary respiration in neonatal dystrophic dogs [44]. In dystrophic dogs, serum CK levels transiently decreased at 3 weeks of age before progressively increasing again until 3 months of age. Serum OPN levels, but not other biomarkers, were also significantly correlated with phenotypic severity in dystrophic dogs at 2 months of age, which corresponded with the onset of muscle weakness. Immunohistologically, OPN expression was observed in infiltrating CD11b- and CD18-positive macrophages or granulocytes, but not in CD3-positive T lymphocytes. OPN was also characteristically expressed in developmental myosin heavy chain (dMyHC)-positive immature regenerating muscle fibers (Fig. 1A). In particular, the number of OPN-positive regenerating muscle fibers increased with active muscle regeneration during the progressive phase but decreased with inactive muscle regeneration during the chronic phase, which was similar to the serum OPN elevation patterns over the disease course. We also noticed that OPN expression, induced by cardiotoxin injection, was detected in serum and muscle during the synergic muscle regeneration process. These observations strongly suggest that OPN is an important mediator of muscle regeneration in the early dystrophic phase. We did not identify any SNPs in $CXMD_J$ analogue to rs28357094 in the human *OPN* gene promoter as a genetic modifier (unpublished).

Next, we compared serum OPN levels with those of both serum MMP-9, a marker of muscle inflammation [46, 71, 104], and its inhibitor, TIMP-1, to confirm whether serum OPN is an indicator of muscle regeneration or inflammation [43]. As a result, the serum MMP-

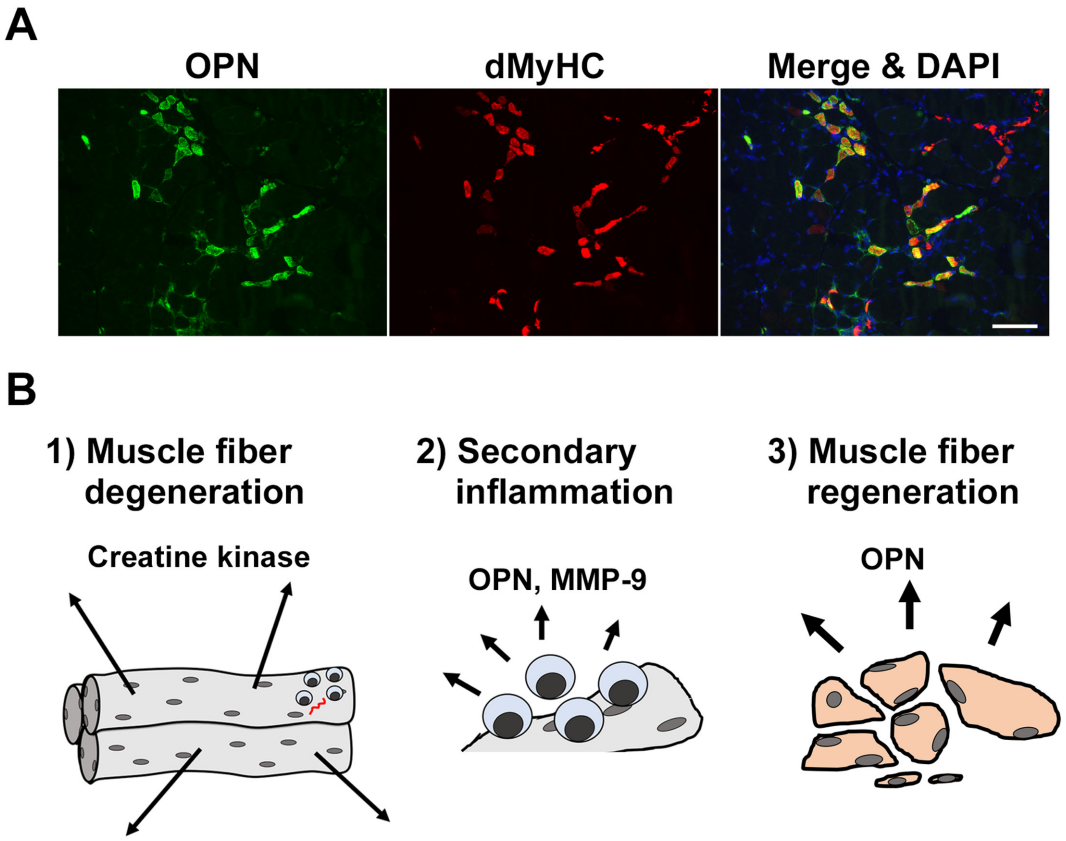

Fig. 1. Osteopontin in the dystrophic pathology. (A) Osteopontin (OPN) expression in regenerating dystrophic muscle fibers. Images are shown of sections of tibialis cranialis muscle of a dystrophic dog immunostained for OPN (green) and developmental myosin heavy chain (dMyHC, red) at 5 months of age. A merged image co-stained with DAPI (blue; nucleus) is shown on the right. Scale bar: 100 μm. (B) Serum biomarkers related to the dystrophic pathology. 1) Muscle fiber degeneration. Muscle fibers are shown as elongated tubes containing many nuclei. Dystrophin-deficient muscle fibers are degenerated because of their fragility with respect to mechanical stress. Creatine kinase has leaked from degenerating muscle fibers, and its serum levels are drastically elevated. 2) Secondary inflammation. Inflammatory cells, including macrophages, are attracted and promote inflammation; OPN and other factors participate in these events. Matrix components are cleared by matrix metalloproteinase (MMP)-9 secreted from macrophages for tissue remodeling. 3) Muscle fiber regeneration. OPN is intensely expressed in regenerating muscle fibers, suggesting its role in muscle regeneration.

9 levels were found to be significantly elevated at 1 h after birth and 3 months of age in dystrophic dogs compared with WT dogs, and the post-birth levels were significantly increased compared with the pre-birth values. MMP-9 expression was immunohistologically observed in CD11b- and CD-18 positive macrophages or granulocytes, but not in dMyHC-positive muscle fibers. Although OPN has been shown to contribute to increased amounts of MMP-9 as an immunomodulator [105], the serum levels of OPN and MMP-9 are not similarly elevated in dystrophic dogs. MMP-9 appears to reflect a rapid inflammatory response by quickly converting the latent form (i.e., the form deposited in the extracellular matrix) to the active form through a proteolytic cascade [106] and immediately being released from storage within infiltrating granulocytes [107]. By contrast, serum levels of OPN and MMP-9 are consistently elevated at 3 months of age, which suggests the active reflection of a serial change in muscle inflammation and regeneration. The elevation pattern of serum TIMP-1 in dystrophic dogs was similar to that of serum OPN, but the levels were not significant compared with those of the WT dogs. TIMP-1 expression was also detected in CD11b- and CD-18-positive mononuclear cells and dMyHC-positive muscle fibers. Considering the similar aspects of OPN and TIMP-1, these two factors may interact with each other in an MMP-independent manner. Indeed, in addition to inhibiting MMP-9, TIMP-1 has been shown to play roles in the processes of cell growth, myogenesis, and fibrosis [72, 108, 109].

We therefore suggest that OPN is a promising biomarker for muscle regeneration in dystrophic dogs [43]. Elevated OPN levels in blood have also been reported in *mdx* mice and GRMD dogs, supporting our results [88, 110]. The serum biomarkers evaluated in our studies are summarized in Fig. 1B.

The perspective of OPN as a serum biomarker for DMD

In patients with DMD, OPN levels in blood are reported to be linearly correlated with muscle function [110]; however, OPN levels in blood were not found to be significantly different between healthy subjects and patients with DMD [71, 110]. A limitation of these previous studies is that they included two DMD cohorts with high mean ages (13.1 ± 6.2 and 16.1 ± 5.6 years), and at those ages, muscle regeneration is not expected to be active. Muscle regeneration in the skeletal muscles of patients with DMD becomes inactive at around 9 years of age [111]. Therefore, serum OPN levels should be analyzed in younger patients with DMD. Serum OPN is also expected to be applicable as an outcome measure in clinical trials with muscle regeneration–inducing agents [112, 113].

Motor Function Evaluations with Accelerometry

Motor function tests in patients with DMD

As patients with DMD progressively manifest abnormal gaits because of muscle weakness, sensitive outcome measures to detect even subtle motor dysfunction are concomitantly needed with the development of new therapeutic methods. Motor function tests, such as the 6-min walk test (6MWT), the timed up and go test, 10-m walk/run velocity, 4-stair climb ascent velocity, and the North Star Ambulatory Assessment, have been applied in clinical assessments of dystrophic conditions [80, 114–116]. The 6MWT, which measures the distance walked in 6 min, is a primary outcome measure of motor function in DMD [117, 118], but this test is not sufficiently sensitive to measure disease progression in younger DMD boys [119]. With the recent development of miniature body-worn motion sensors, accelerometry has become an efficient method to quantify performance in validated tasks and activities of daily living [120–122]. Acceleration parameters indicating the movement and orientation of the body and upper limbs have been measured to assess physical activities involving both ambulatory and nonambulatory conditions in patients with DMD [123–126]. Accelerometry has also been used to monitor disease progression and the effects of corticosteroid treatment for informal tasks such as walking [127, 128]. When combined with different types of information, such as angular velocity, accelerometry has been shown to be more practical for capturing motion [122, 126, 127].

Accelerometric outcomes in $CXMD_J$

Accelerometry has been performed in GRMD dogs, and their acceleration parameters during their gaits have been found to be attenuated over the disease course [129–131]. In our study, gait was longitudinally evaluated in $CXMD_J$ by measuring acceleration and angular velocity parameters that have been used to assess the relationship with clinical evaluations in dystrophic dogs [132]. We used portable wireless hybrid sensors (TSND121, ATR-Promotions, Inc., Soraku-gun, Kyoto, Japan) to measure three-axis acceleration and angular velocity values within a wide range: ± 8 G and ± 1,000 degrees per second, respectively. These miniature sensors (46 × 37 × 12 mm; weight 22 g) were affixed on the dorsal thoracic and lumbar regions of the dogs. The three axes were the X-axis (caudal–cranial), Y-axis (medial–lateral), and Z-axis (ventral–dorsal). The specific acceleration vector indicates the instantaneous inertial acceleration for each axis (*Ax*, *Ay*, and *Az*, respectively). The instantaneous angular velocity vector indicates the instantaneous rotation of the trunk (*Gx*, *Gy*, and *Gz*, respectively). The three-axis acceleration and angular velocity vectors on the dog subjects are summarized in Fig. 2. These data were recorded from trials in which the dogs ran down a 15-m hallway four times. These trials and clinical tests were performed in five $CXMD_J$ dystrophic dogs and six WT dogs ranging from 2 to 12 months of age.

The dystrophic dogs showed a bunny hop at the stance and swing phases during gallop and changed their gait pattern to a trot or walk according to the severity. The instantaneous vectors of acceleration (*Ax*, *Ay*, *Az*) were calculated as the average of the absolute values for each axis. The results showed that all three-axis vectors were lower in dystrophic dogs than in WT dogs in both the dorsal thoracic and lumbar regions and were progressively attenuated over the disease course. The acceleration magnitude (*AM*) was then calculated from the three acceleration vectors (*Ax*, *Ay*, *Az*) as the square root of the sum of the three-axis values ($AM = \sqrt{Ax^2 + Ay^2 + Az^2}$) and averaged for each trial. The results showed that the *AM*s, especially those in the thoracic region, were already notably lower in dystrophic dogs than in WT dogs at 2 months of age, which is when the onset of muscle weakness occurs in dystrophic dogs. *AM*s in the lumbar region were drastically attenuated over the disease course in dystrophic dogs. These results demonstrate differences in *AMs* between the dorsal thoracic and lumbar regions. It has been reported that forelimb and hindlimb gait movements strongly influence thoracic and lumbar motion, respectively [133]. In the standing position, the load in the vertical direction is more strongly

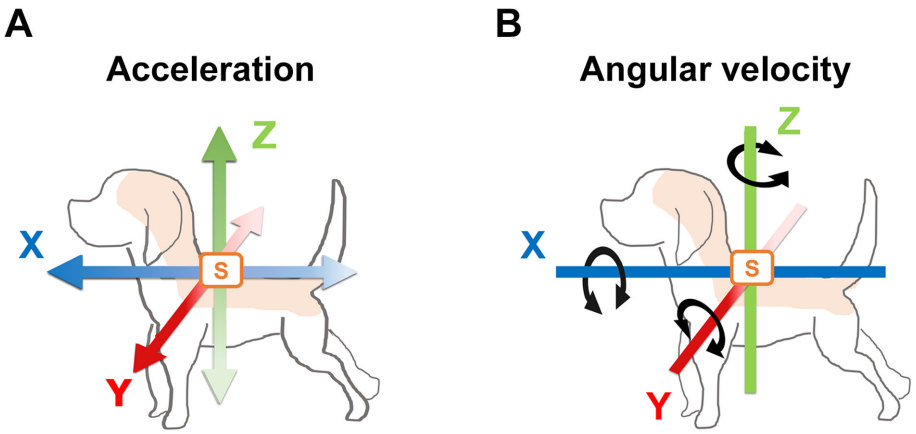

Fig. 2. Illustrations of the three axes of acceleration and angular velocity in a dog subject. Wireless sensors (indicated by S in orange) are affixed to the dorsal region of a dog to detect three-axis acceleration and angular velocity values. The three axes are indicated as the X-axis (blue; caudal–cranial), Y-axis (red; medial–lateral), and Z-axis (green; ventral–dorsal). (A) Three axes of acceleration are shown with colored bidirectional arrows, including the inertial instantaneous acceleration for each axis. Dense colored arrows for the X-, Y-, and Z-axes indicate the fore, left, and upper directions, respectively, as vectors with positive values, while pale colored arrows indicate the opposite directions as vectors with negative values. (B) Three axes of angular velocity are shown with bidirectional arrows and indicate the instantaneous rotation of the colored bars for each axis. Clockwise rotation indicates vectors with positive values, while the opposite rotation indicates vectors with negative values.

applied to the forelimb than to the hindlimb, with a ratio of at least 6:4 [133–137], which suggests that the forelimbs are mainly loaded from the early stage at the initiation of walking. Owing to the strut load, dystrophic dogs might experience muscle involvement, especially in the forelimb, before the onset of muscle weakness, which could lead to reduced acceleration in the thoracic region. Thus, the drastic decline in *AM* in the lumbar region is likely related to the progressive involvement of the hindlimb muscles during the disease course.

We also analyzed the acceleration ratios (*Ax* ratio, *Ay* ratio, *Az* ratio), which are the relative components of the *AM* along the three axes (%), to detect three-axis biases for acceleration as a whole. The acceleration ratios were calculated by dividing the absolute values of each axis by the *AM* and then averaged in each trial. The *Ax* ratios in the dorsal thoracic and lumbar regions were lower in dystrophic dogs than in WT dogs. In dystrophic dogs, the *Ay* ratio progressively increased in the thoracic region over the disease course, whereas that in the lumbar region slightly increased at 10 and 11 months of age. By contrast, the *Az* ratio was slightly higher in the lumbar region in dystrophic dogs than in WT dogs at 8 months of age. The attenuation of the *Ax* ratio in dystrophic dogs reflects a progressive decay in the forward propulsive force, whereas the increase in the *Ay* and *Az* ratios might be indicative of a heightening motion potentially related to a waddling-like gait and a bunny hop, respectively.

The instantaneous vectors of angular velocity (*Gx*, *Gy*, *Gz*) were calculated as the average of the absolute values of each axis. Three-axis vectors were lower in dystrophic dogs than in WT dogs in both the dorsal thoracic and lumbar regions and, except for *Gz* in the lumbar region, were found to be attenuated over the disease course. *Gy* in the thoracic region was substantially increased in WT dogs and drastically different from that in dystrophic dogs. Limb behavior during the gait of dogs causes a wider sweeping motion of the thorax because of forelimb movement in the horizontal and vertical axes [133]. In WT dogs, an increase in *Gy* in the thoracic region reflected dynamic forelimb motion during a gallop; therefore, greater power was applied to that leg. By providing a strong load in the early phase, the intense motion of the thorax in dystrophic dogs resulted in forelimb muscle involvement, which, in turn, led to gait dysfunction and a reduced *Gy* in the thoracic region. *Gz* in the lumbar region was lower in dystrophic dogs compared with WT dogs, but it showed an increase to that in WT dogs at 8 months of age. This observation could be the result of waddling at the girdle, which suggests the importance of motion evaluation in the lumbar region.

Multiple parameters of acceleration and angular velocity were correlated with the clinical manifestations, as determined using a grading scale of clinical signs in $CXMD_J$, which was described earlier. The coefficients of the total grading scores for multiple parameters were less than 0, except for *Gz* in the lumbar region. These findings revealed that a number of parameters mostly

decreased with exacerbated severity, in contrast to *Gz* in the lumbar region, which increased. Among the acceleration ratios, the coefficient for the *Ay* ratio in the thoracic region was greater than 0, indicating that acceleration in the medial–lateral direction in the thoracic region increased with exacerbated severity. These findings suggest that an increased *Gz* in the lumbar region and a higher *Ay* ratio in the thoracic region are associated with a waddling-like gait in the severe phenotype of $CXMD_J$.

From these results, multiple parameters of acceleration and angular velocity can be considered remarkably sensitive for evaluating pathological conditions. We found that these parameters also increased in association with high spontaneous locomotor activity in dystrophic dogs [132], implying a relationship between activities of daily living and motor dysfunction similar to that in patients with DMD [124, 125, 128]. Cell therapies such as MSC treatment in dystrophic dogs have been shown to ameliorate the acceleration parameters [62]. Accelerometry has been found to provide quantitative, multifaceted kinematic indices in combination with conventional motor function tests in clinical trials.

Conclusion

We investigated serum OPN expression and accelerometry in $CXMD_J$ to develop outcome measures for the clinical evaluation of DMD. The results in $CXMD_J$ suggested that serum OPN is a promising biomarker for muscle regeneration and that multiple acceleration and angular velocity parameters are also efficient outcome measures for the quantification of motor function according to the disease course and severity. It is necessary to carefully examine these outcome measures in patients with DMD, which shows different pathological conditions depending on symptom onset and disease phase from those of canine dystrophic models [28, 138]. It is also necessary to define the accelerometric characteristics of dystrophic quadrupedal gaits and to cautiously extrapolate this information to the bipedal gaits of patients. These outcome measures would be potentially applicable to patients with hereditary neuromuscular disorders, including DMD.

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