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A pilot study on nitration/dysfunction of NK1 segment of myogenic stem cell activator HGF

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ABSTRACT

Protein tyrosine residue (Y) nitration, a post-translational chemical-modification mode, has been associated with changes in protein activity and function; hence the accumulation of specific nitrated proteins in tissues may be used to monitor the onset and progression of pathological disorders. To verify the possible impact of nitration on postnatal muscle growth and regeneration, a pilot study was designed to examine the nitration/dysfunction of hepatocyte growth factor (HGF), a key ligand that is released from the extracellular tethering and activates myogenic stem satellite cells to enter the cell cycle upon muscle stretch and injury. Exposure of recombinant HGF (a hetero-dimer of α- and β-chains) to peroxynitrite induces Y nitration in HGF α-chain under physiological conditions. Physiological significance of this finding was emphasized by Western blotting that showed the NK1 segment of HGF (including a K1 domain critical for signaling-receptor c-met binding) undergoes nitration with a primary target of Y198. Peroxynitrite treatment abolished HGF-agonistic activity of the NK1 segment, as revealed by *in vitro* c-met binding and bromodeoxyuridine-incorporation assays. Importantly, direct-immunofluorescence microscopy of rat lower hind-limb muscles from two aged-groups (2-month-old "young" and 12-month-old "retired/adult") provided *in vivo* evidence for age-related nitration of extracellular HGF (Y198). Overall, findings provide the insight that HGF/NK1 nitration/dysfunction perturbs myogenic stem cell dynamics and homeostasis; hence NK1 nitration may stimulate progression of muscular disorders and diseases including sarcopenia.

1. Introduction

Protein tyrosine nitration represents an oxidative post-translational modification. Due to the incorporation of a highly bulky nitro group in the tyrosine phenolic ring, nitration modifies the physicochemical properties of tyrosine residues (Ys) and hence alters the activity and function of their target proteins [1–4]. Tyrosine nitration does not occur at random (not all proteins undergo nitration); accumulation of specific nitrated proteins in particular tissues and organs thus, has potential to be a biomarker for the onset and progression of several disorders and diseases including cardiovascular damage [5,6], colon and lung cancers [7–9], and two neurodegenerative disorders, Alzheimer's and Parkinson's diseases [10,11]. Nitration is induced *in vivo* by peroxynitrite ($ONOO^-$), a highly reactive biomolecular with a very short half-life formed by the rapid reaction of nitric oxide (NO) and superoxide ($O_2^{\cdot-}$) radicals. Therefore, the accumulation of nitrated proteins is mainly reliant upon the peroxynitrite formation sites [1,2,12,13].

Skeletal muscle is particularly susceptible to the effects of peroxynitrite as it is an organ that continuously generates reactive oxygen and nitrogen species, even under physiological conditions and definitely

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heightens their generation in pathologic disorders [14–18]. Skeletal muscles display a high regenerative capacity that depends on sequential dynamics of myogenic stem satellite cells, which are normally quiescent or dormant and respond to activation signals to enter the cell cycle [19–21] in a NO-radical-hepatocyte growth factor (HGF)-dependent manner [22–26]. The regenerative function of satellite cells is orchestrated by their interaction with a complex of regulatory microenvironmental factors to adapt skeletal muscle function to physiological demand and metabolic changes [27,28]. It is well recognized that muscle regeneration after a minor injury such as strain, is completed spontaneously and more rapidly than after an extensive crush injury in which incomplete healing with fibrosis and calcification lesions impairs muscle function [29,30]. We speculated that the significant delay (or disruption) of muscle regeneration after traumatic damage may be associated with pathological up-regulation of peroxynitrite formation and subsequent protein-tyrosine nitration that may disrupt the sequential dynamics of satellite cells.

HGF [31,32], also known as scatter factor, is a pleiotropic multidomain-protein in the plasminogen subfamily. The active form of HGF is a heterodimer of α- and β-chains previously demonstrated to activate quiescent satellite cells to enter the cell cycle in postnatal muscle growth and regeneration through its release from extracellular tethering and presentation to the cell-membrane receptor c-met [23,24,33–35]. HGF α-chain (about 60 kDa) is composed of a *N*-terminal domain (also called the PAN/APPLE-like domain) followed by four Kringle domains (K1 to K4), and originally linked to a 30-kDa β-chain subunit (Fig. S1 panel A). NK1, a sequence of *N*-terminal and K1 domains, is a critical segment for the biological activity of HGF because K1 has essential residues (including Y198 in the human protein, highly conserved among mammalian species) for a high-affinity binding site to c-met; as well, the *N*-terminal domain may participate in K1-c-met interaction [36–38]. HGF lacking NK1 is unable to activate c-met [39], indicating again the physiological significance of that segment. These observations suggest the hypothesis that if the NK1 segment may undergo nitration, the change will inhibit satellite cell activation.

To test the above hypothesis, we conducted preliminary *in vitro* experiments using peroxynitrite treatment of recombinant HGF and NK1 under physiological conditions. Results clearly demonstrated that functionally important Y198 residue in the K1 domain undergoes nitration and accounts for important results supporting that the nitration may abolish c-met binding and bromodeoxyuridine (BrdU)-incorporation activities of NK1. Physiological significance of this finding was supported by direct-immunofluorescence microscopy for rat lower hindlimb muscles, extensor digitorum longus (EDL) and tibialis anterior (TA) muscles from 2-month-old "young" and 12-month-old "retired/adult" groups; results showed that *in vivo* nitration of extracellular HGF (Y198) was detected more pronouncedly at 12-months old of the early aging-phase. Findings may provide biomedical strategies to combat dysfunction induced by HGF/NK1 nitration in age-related muscular disorders and diseases.

2. Materials and methods

2.1. Materials

Recombinant mouse HGF (2207-HG/CF; carrier-free; a disulfide-linked heterodimer of α- and β-chains as a major form in the product) was purchased from R&D Systems (Minneapolis, MN, USA) and recombinant human NK1 (22.5 kDa; 32Q-210E with an additional sequence CHHHHHH-PRAAAVKSP at *N*-terminal; see Fig. S1 panel A) was a kind gift from TORAY (Tokyo, Japan). Peroxynitrite (P332) was purchased from Dojindo Laboratories (Kumamoto, Japan). Horseradish peroxidase (HRP)-conjugated monoclonal anti-nitrotyrosine (clone 39B6; sc-32757 HRP), HRP-conjugated monoclonal anti-human HGF α-chain (clone H-10, sc-374422 HRP), and Alexa Flour 594-labeled monoclonal anti-human HGF α-chain antibodies (clone H-10, sc-374422 AF594) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Recombinant human superoxide dismutase 1 (SOD1; PRO-648) was purchased from ProSpec-Tany Technogene (Rehovot, Israel). Monoclonal anti-nitrotryptophan antibody (clone 117C, JAI-MNW-020P) was from Bio-Connect Life Sciences (Huissen, Netherlands). Polyclonal anti-human HGF neutralizing antibody (AB-294-NA) was purchased from R&D Systems and HRP-conjugated donkey anti-goat IgG (705-036-147; $F(ab')_2$ fragment affinity-purified) was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Rat monoclonal anti-nitrated Y198 HGF-specific antibody was designed and raised in-house against a synthetic peptide consisting of 12 amino acids including nitrated Y198 and the immuno-specificity has been evaluated by ELISA of nitrated/non-nitrated immunogens and mouse whole-HGF protein and by Western blotting of nitrated/non-nitrated HGF and NK1 segment and nitrated bovine serum albumin (BSA) under reducing conditions (See Figs. 2B and S2; detailed information is available soon; manuscript in preparation by the authors). HRP-labeled rabbit anti-rat IgG was purchased from Abcam (ab6734; Cambridge, MA, USA; antigen-affinity purified). Amersham enhanced chemiluminescence (ECL) detection kit (PRN2106) and nitrocellulose membranes (10600048) were purchased from GE Healthcare (Little Chalfont, UK).

Dulbecco's modified Eagle's medium (DMEM; 31600-034), horse serum (HS; 16050-122), antibiotic-antimycotic (15240-062), and gentamicin (15710-064) were all from Invitrogen (Grand Island, NY, USA). Poly-L-lysine (P-9155), bovine plasma fibronectin (F1141), protease type XIV (P5147), 5-Bromo-2'-deoxyuridine (BrdU; B5002), and HRP-labeled goat anti-mouse IgG antibody (A4416) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal anti-BrdU (clone G3G4) and anti-desmin antibodies (clone D3) were from Developmental Studies Hybridoma Bank (Iowa City, IA, USA).

Recombinant mouse HGFR/c-met Fc chimera (7065-ME; carrier-free), biotinylated polyclonal anti-human HGF antibody (BAF294; antigen affinity-purified), streptavidin-HRP conjugate (4800-30-06), and tetramethylbenzidine (TMB) substrate solution (DY999) were purchased from R&D systems.

2.2. Protein nitration

A peroxynitrite stock solution (0.1 M in 0.3 M NaOH–NaCl) was diluted to less than 1/100 with the addition of ice-cold deionized water. The nitration reaction was then initiated by quick addition of the dilute peroxynitrite solution to either recombinant HGF or NK1 under physiological conditions (pH 7.4 at 37°C for 30 min in PBS) at different molar ratios of protein to peroxynitrite.

2.3. ECL-Western blotting

Tyrosine nitration was visualized by subjecting peroxynitrite-treated proteins to sodium dodecyl sulfate (SDS)-12% PAGE and transfer to nitrocellulose membranes [40]. Briefly, the blots were incubated overnight at 4°C with HRP-conjugated anti-nitrotyrosine antibody (1:2500 dilution in CanGetSignal solution 1; NKB-101, Toyobo, Osaka, Japan), followed by ECL-detection on a FUSION SOLO.7S.EDGE imaging system (Vilber Lourmat, Marne-la-Vallée, France). Subsequently, the blots were agitated for 45 min at 50°C in stripping buffer (2% SDS, 0.8% β-mercaptoethanol (ME), and 62.5 mM Tris-HCl, pH 6.8) and then re-probed with a set of polyclonal anti-HGF (1:5000 dilution) and HRP-conjugated anti-goat IgG antibodies, or with HRP-labeled anti-HGF antibody to monitor HGF on the blots as loading controls.

Further immunoblot analyses were carried out by using anti-nitrated Y198-HGF and HRP-conjugated anti-rat IgG antibodies. After ECL detection, conjugated HRP was inactivated by 30% H_2O_2 treatment of the blots (for 30 min at room temperature) [41] to proceed to the second round of immunoreaction without chemical-stripping of the blots by HRP-conjugated anti-nitrotyrosine antibody (overnight at 4°C) to see if

tyrosine residues in the NK1 segment other than Y198 were nitrated.

2.4. Animal care and use

All experiments involving animals were conducted in strict accordance with the recommendations in the Guidelines for Proper Conduct of Animal Experiments published by the Science Council of Japan and ethics approvals from the Kyushu University Institutional Review Board (No. A30–143 and A20-014).

2.5. Satellite cell isolation and primary culture

Satellite cells were isolated from muscle in the upper hind limb and back of adult male Sprague-Dawley rats (9 to 12-month-old) [22,42] with a slight modification [43]. Briefly, muscles were excised, trimmed of connective tissue and fat, minced with sterile scissors, and digested with 1.25 mg/ml of protease type XIV for 1 h at 37°C. Cells were separated from muscle tissue debris by differential centrifugation and filtration through nylon cell strainers (100 μm and 40 μm mesh size) prior to a final centrifugation step at 1500×*g* for 3 min. Cells were suspended in DMEM containing 10% HS, 1% antibiotic-antimycotic mixture, and 0.5% gentamicin (DMEM-10% HS) and plated on poly--L-lysine and fibronectin-coated cluster-dishes.

Cultures were maintained in a humidified atmosphere of 5% CO_2 at 37°C for 24 h and then incubated for the next 24-h period in DMEM-10% HS additionally containing control or nitrated HGF/NK1 at 3.3×10^{-5} μM (3 ng/ml HGF, 0.74 ng/ml NK1) or 11×10^{-5} μM (10 ng/ml HGF, 2.48 ng/ml NK1) of the final concentrations in culture media; peroxynitrite treatment was done at 1:4000 of protein: peroxynitrite molar ratio at pH 7.4 for 30 min in this case.

Cultures were pulse-labeled with 10 μM BrdU in DMEM-10% HS for the final 2 h at 48 h post-plating (fixation time-point), followed by immunocytochemistry for detection of BrdU using a G3G4 anti-BrdU monoclonal antibody (1:100 dilution in 0.1% BSA in PBS) and an HRP-conjugated anti-mouse IgG antibody (1:500 dilution) according to Tatsumi et al. [23]. The mean percentage of BrdU-labeled cells for three cultures per treatment was used as an indicator of activation (entry into the cell cycle) and the subsequent proliferation activity of satellite cells. In addition, companion satellite-cell cultures, prepared at the same time, were immunostained for the presence of desmin at 30 h after plating, using a D3 anti-desmin monoclonal antibody in order to determine the percentage of myogenic cells present [22,33,34]; cultures with less than 95% 3,3'-diaminobenzidine (DAB)-positive cells were not used for experiments.

2.6. c-Met binding assay

According to Tatsumi et al. [34] with some modifications, 96-well ELISA plates were coated with mouse c-met-Fc chimera overnight at room temperature by adding 50 μl of 1 μg protein/ml in PBS to each well, which was then blocked for 2 h at room temperature with 1% BSA, 5% sucrose, and 0.05% sodium azide in PBS (150 μl/well). Plates were washed with 0.1% polyethylene sorbitan monolaurate (Tween 20)-Tris--buffered saline (TTBS) and incubated in CO_2-incubator for 2 h with 50 μl/well of 0.003 μM and 0.015 μM NK1 treated with peroxynitrite at various molar ratios (1:0, 1:100, 1:2000, and 1:4000 in PBS, pH 7.4). Regular HGF (0.003 μM; without peroxynitrite treatment) was assigned to a positive control sample. After TTBS wash and fixation with 3.7% (v/v) paraformaldehyde in PBS for 5 min at 4°C, each well was refilled with 150 μl blocking solution overnight at 4°C. The binding of NK1 to c-met was detected with biotinylated anti-HGF antibody (1:500 dilution in TTBS, for 3 h at room temperature) and HRP-streptavidin (1:1600 dilution in TTBS, for 20 min at room temperature). After a final wash with TTBS, 100 μl of tetramethylbenzidine substrate solution was dispensed to wells; the color development was stopped by 1 M sulfuric acid solution (25 μl/well), followed by optical density measurements at wavelengths of 450 and 530 nm.

2.7. Direct-immunofluorescence microscopy

A muscle group of extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of lower hindlimb was dissected from "young" (2-month-old) and "retired/adult" (12-month-old) Sprague-Dawley rats ($n = 3$ per age group), oriented in tissue OCT compound, and then frozen in isopentane cooled with liquid nitrogen. Cryo-sections (about 13-μm thickness) were prepared using a Leica CM1950 cryostat (Nussloch, Germany) and examined for nitration of extracellular matrix (ECM)-bound HGF by direct-immunofluorescence microscopy. In brief, according to Sawano et al. [44] with a slight modification, muscle cross-sections were fixed with hot PBS and steam for 5 min and blocked with sterile donkey-serum solution (containing 2% normal serum, 1% BSA, 0.1% cold fish skin gelatin, 0.05% Tween 20, 0.01% avidin, 100 mM glycine, and 0.05% sodium azide in PBS, pH 7.2) for 1 h at 25°C before incubation overnight at 4°C in Fluorescein 500-labeled anti-nitrated Y198 HGF monoclonal antibody raised in-house (1:50 dilution in the sterile solution containing 1% BSA, 0.1% cold fish skin gelatin, 0.05% Tween 20, and 0.05% sodium azide in PBS). Another set of serial cryo-sections was stained with Alexa Fluor 594-labeled anti-HGF monoclonal antibody (1:50 dilution) overnight at 4°C. Sections were mounted in VECTASHIELD Antifade Mounting Medium (Vector Lab., Burlingame, CA, USA) and observed under a Leica DMI6000B-AFC fluorescence microscope equipped with a DFC365FX digital camera and LAS AF 3.1.0 software that controls a Tile-Scan program.

2.8. Statistical analyses

Student's *t*-tests were employed for statistical analysis of experimental results using Microsoft Excel X (Figs. 3 and 4). Data are represented as mean ± standard error (S.E.) for 3 cultures or wells per treatment. The level of significance was set to $p < 0.05$ and statistically significant differences between two groups at $p < 0.05$, $p < 0.01$, and $p < 0.001$ are indicated throughout by (*), (**), and (***), respectively. Results are representative examples of more than two or three independent experiments.

3. Results

3.1. Nitration of HGF α-chain

To assess the nitration impact of key growth factors on regenerative myogenesis, we first examined whether the myogenic stem satellite cell activator HGF undergoes nitration under physiological conditions. Recombinant mouse HGF was exposed to peroxynitrite at pH 7.4 and evaluated for nitration status by ECL-Western blotting (Fig. 1). The results clearly showed that anti-nitrotyrosine antibody reacted with the 90-kDa proform of HGF (not yet cleaved into α- and β-chains) and 60-kDa α-chain; the antibody did not show significant immuno-reactivity to 30-kDa β-chain, which was visualized after chemical stripping of the blot (SDS-βME wash) and reprobing with anti-HGF antibody. These results indicate that the HGF α-chain is essentially the recipient of nitration activity targeting Ys.

3.2. NK1 nitration with a primary target of Y198

To further define and characterize the susceptibility of HGF for nitration, subsequent experiments were conducted using recombinant NK1 that is the *N*-terminal segment of HGF responsible for binding to the receptor c-met (Fig. 2A). Western blots again showed nitration of peroxynitrite-treated HGF (lane 1, the positive control) in contrast to the negative control (lane 2, peroxynitrite-untreated HGF). A faint band of β-chain (and/or a degradation product of α-chain; marked by asterisk)

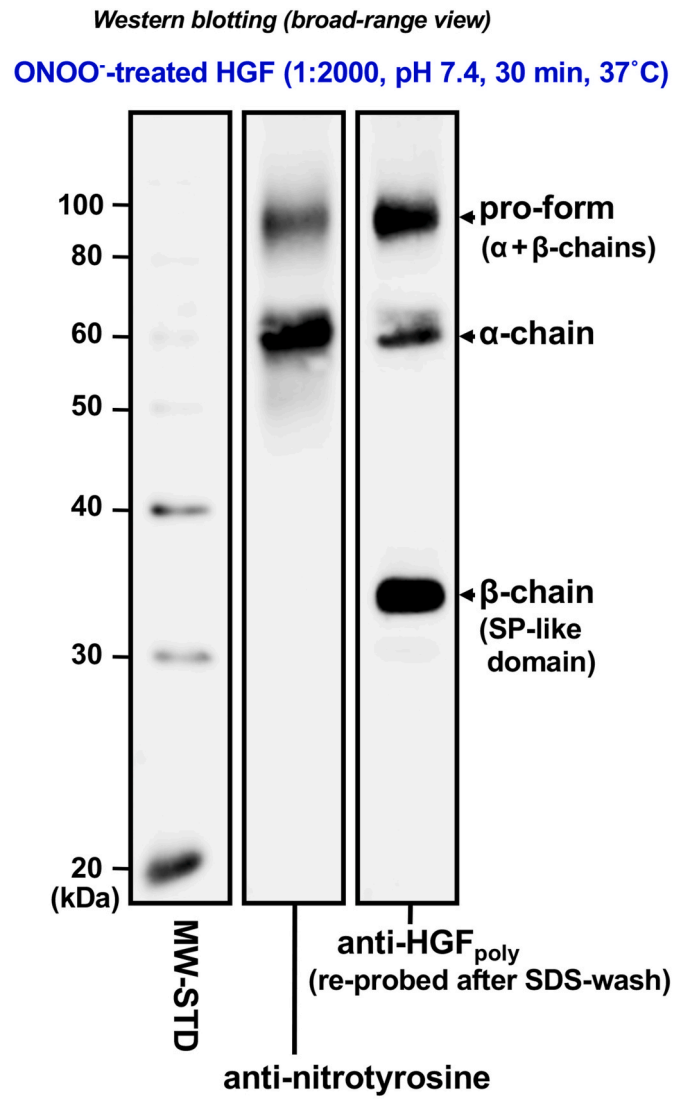

Fig. 1. HGF α-chain undergoes tyrosine nitration by peroxynitrite. Recombinant mouse HGF was incubated with peroxynitrite (at 1:2000, pH 7.4, 37°C, for 30 min) and evaluated for nitration status by Western blotting. MW-STD (lane 1), molecular weight standards (Magic Mark); lane 2, treated with HRP-labeled anti-nitrotyrosine antibody; lane 3, stripped with SDS-β-ME solution and re-probed with anti-HGF antibody.

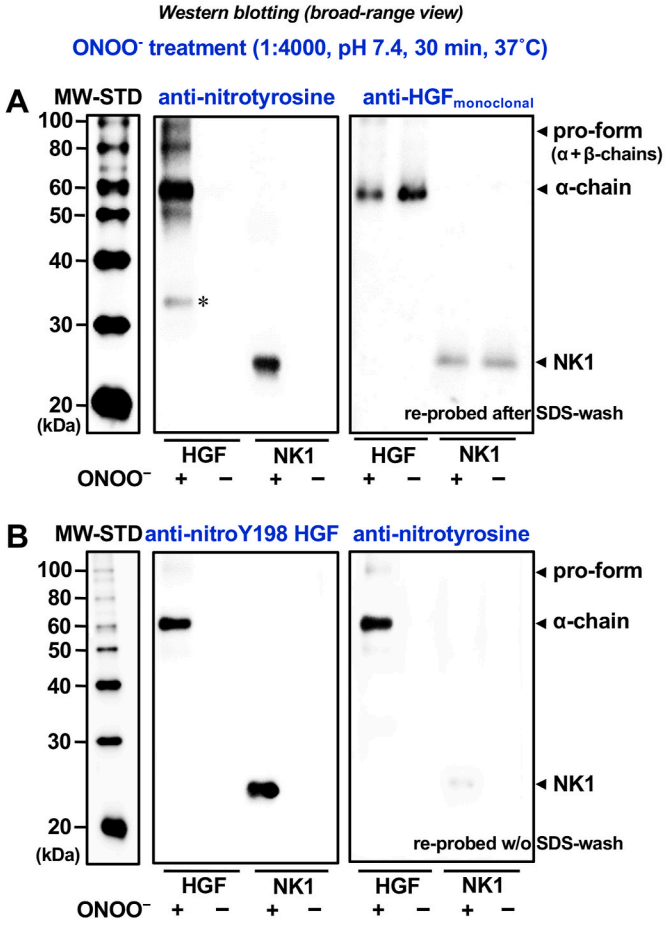

Fig. 2. NK1 nitration with a primary target of Y198. Recombinant HGF and NK1 were incubated with (+) or without (−) peroxynitrite ($ONOO^-$) and visualized for nitration status by Western blotting. MW-STD (far-left blot), molecular weight standards (Magic Mark). (**A**) A blot treated with HRP-labeled anti-nitrotyrosine antibody (left column), followed by stripping with SDS-β-ME and re-probing with HRP-labeled anti-HGF α-chain antibody as a loading control (right column). *, A faint band of β-chain and/or a degradation product of α-chain (lane 1). (**B**) A different set of Western blots including a blot treated with anti-nitrated-Y198-HGF antibody (left column), followed by re-probing directly with HRP-labeled anti-nitrotyrosine antibody (right column; without striping the anti-nitrated-Y198).

was also seen along with immuno-positive 80-kDa and 50-kDa fragments, all of which were negligible and therefore beyond the scope of this study (lane 1). Peroxynitrite-treated NK1 segment (22.5 kDa) was clearly recognized by probing with the anti-nitrotyrosine antibody (lane 3), in contrast to undetectable immuno-reactivity of untreated control NK1 (lane 4). Loading-protein amounts on the blots were comparable between the peroxynitrite-treated and untreated-control lanes, as revealed by reprobing the membrane with anti-HGF α-chain antibody (Fig. 2A, right column). These results indicate that the NK1 segment in HGF α-chain undergoes nitration of Ys in response to peroxynitrite treatment.

It is worth noting that highly specific Y(s) may receive nitration according to the structural attributes of a target protein [45]. Based on the amino acid sequence of NK1 (Fig. S1 panel A) and the accessible surface area calculated for Cε atoms of the tyrosine side-chain in a 3D-model of NK2 segment (PDB ID: 3SP8 [46]), Y198 was expected to be a most preferential nitration target in the NK1 segment. This issue was assessed by immunoblot analysis with anti-nitrated Y198 HGF-specific antibody. The results are shown in Fig. 2B left panel, in which peroxynitrite attacked Y198 of HGF and NK1 as revealed by immuno-detection of nitrated Y198 (lanes 1 and 3), while no band was detected for controls that were not treated with peroxynitrite (lanes 2 and 4).

The subsequent experiment was performed to examine whether tyrosine residues other than Y198 in the NK1 segment are nitrated, although analyses for the amino acid sequences neighboring individual Y(s) (five in total, in addition to Y198) did not support this possibility. After the first run of Western blotting with anti-nitrated Y198 HGF, blots were incubated in 30% H_2O_2 to irreversibly inactivate the antibody-conjugated HRP; this was followed by reprobing with anti-nitrotyrosine antibody that can recognize all nitrated tyrosine residues without stripping the anti-nitrated Y198 antibody. As shown in Fig. 2B right column, immuno-reaction intensity of nitrated Y198 was drastically decreased down to a faint level comparable to the peroxynitrite-untreated control NK1 (lanes 3 and 4). In contrast, whole HGF protein again displayed intense immuno-response to anti-nitrotyrosine antibody on the same membrane, and therefore served as a reliable positive control (lane 1). Interestingly, the HGF band intensity appeared to be equivalent to the intensity in the first round of probing using anti-

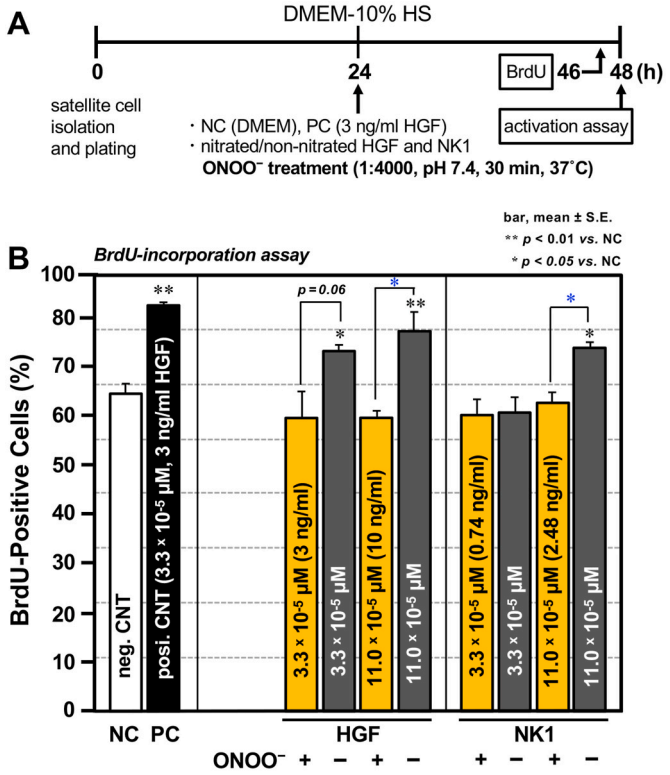

Fig. 3. Dysfunction of HGF and NK1 upon their nitration. (**A**) The experimental scheme. Cell cultures received nitrated or non-nitrated HGF or NK1 at 24-h post-plating and were pulse-labeled with BrdU for 2 h just prior to fixation at 48-h. (**B**) PC, positive control culture with HGF at final concentration of 3.3×10^{-5} µM (3 ng/ml in DMEM-10% HS) (black bar). NC, negative control culture in DMEM-10% HS (open bar). Non-nitrated (gray bars) and nitrated HGF/NK1 (orange bars) were added to cultures at 3.3×10^{-5} µM and 11.0×10^{-5} µM in media and assayed for the cell activation activity (percent BrdU-positive cells) by immunocytochemistry. Bars represent mean ± standard error (S.E.); significant differences from the negative control (open bar) at $p < 0.05$ and $p < 0.01$ are indicated by black asterisks (*) and (**), respectively (Student's *t*-tests). Blue single-asterisk indicates a significant difference ($p < 0.05$) between non-nitrated and nitrated proteins.

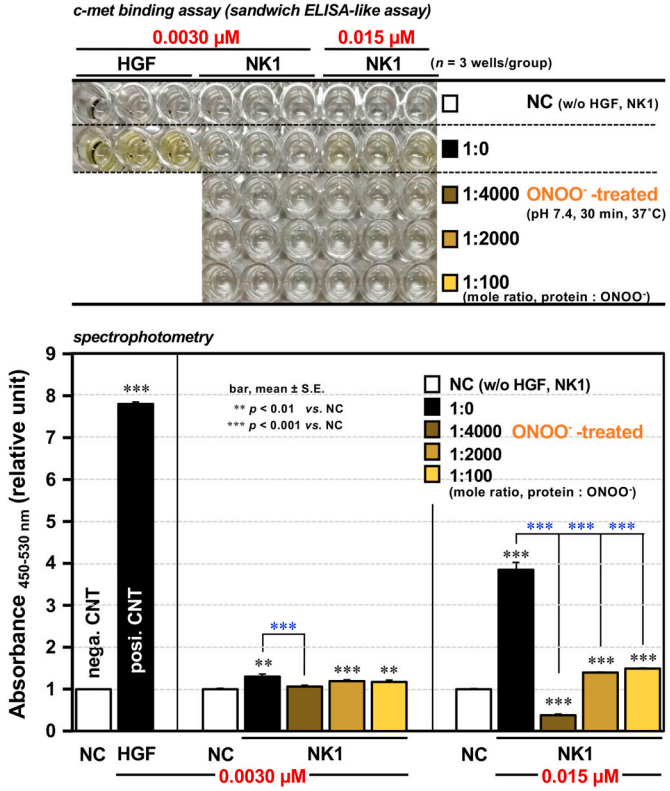

Fig. 4. NK1 nitration abolishes its affinity to the receptor, c-met. Receptor binding activities of HGF (0.0030 µM) and NK1 (0.0030 µM and 0.015 µM), peroxynitrite-treated at different molar ratios) were assayed by sandwich ELISA-like assay on solid-phase of recombinant c-met-Fc chimera (see upper photo from triplicate assays). Optical density was measured at 450 and 530 nm and presented as a relative unit to the negative control (NC; open bars). Non-nitrated HGF (0.0030 µM) served as a positive control (posi. CNT, black bar in the far-left panel). Bars represent mean ± S.E.; significant differences from the respective negative control in each panel (NC, open bars) at $p < 0.01$ and $p < 0.001$ are indicated by black asterisks (**) and (***), respectively. Blue triple-asterisks indicate significant differences between non-nitrated and nitrated NK1 at $p < 0.001$.

nitrated Y198 antibody (see panel B, each lane 1 in the left and right columns), indicating that an additional tyrosine-nitration site (other than Y198) may be localized in domains K2–K4 of the HGF α-chain. Nonetheless, results demonstrated that the NK1 segment of HGF undergoes tyrosine-nitration with a primary target of Y198 under physiological conditions.

3.3. Dysfunction of HGF and NK1 upon peroxynitrite treatment

Nitrated NK1 segment was evaluated for HGF-agonistic activity to activate quiescent myogenic stem satellite cells, in order to further understand the physiological consequences of HGF nitration. Since our previous studies showed that HGF activates satellite cells with maximum effect in the range of 2.5-10 ng/ml in primary cultures [34,47], this study was configured to use two different treatment doses of HGF/NK1for satellite cell activation cultures (3.3×10^{-5} and 11×10^{-5} µM HGF/NK1, equivalent to 3 and 10 ng/ml HGF and 0.74 and 2.48 ng/ml NK1, respectively) along with a positive control with 3 ng/ml regular HGF (black bar, Fig. 3). Primary satellite cell cultures were administrated with either nitrated or non-nitrated control HGF/NK1 for 24 h beginning at 24-h post-plating and assayed for BrdU incorporation to compare the activation activities of satellite cells (see Fig. 3A for the culture scheme). As shown in Fig. 3B, the percentage of BrdU-positive cells in cultures treated with nitrated-HGF (orange bars in mid column) decreased down to a level comparable to the negative-control culture (far-left blank bar) at both doses of HGF. A similar result was obtained in culture exposed to nitrated NK1 at 11×10^{-5} µM, the same molar dose as 10 ng/ml HGF culture which demonstrates maximum activation activity of satellite cells (Fig. 3B, far-right orange and gray bars in the right column). At the lower dose of non-nitrated NK1 (3.3×10^{-5} µM), BrdU-incorporation activity was not elicited to a significant level, and was comparable to the culture with the same molar dose of control HGF, possibly due to lower c-met binding affinity of the NK1 segment that lacks Kringle 2–4 domains and hence co-operative contributions to maintaining the c-met binding integrity [48,49]. We used here, a recombinant NK1 that enrolls an additional 16-amino acid sequence at the *N*-terminal (Fig. S1 panel A), which might affect the c-met binding affinity. Nonetheless, results indicate that HGF and the agonist NK1 lose the key function to activate quiescent satellite cells upon their nitration. Taken together with the results in Fig. 2B, findings provide a possible insight that Y198 nitration in the NK1 segment leads to dysfunction of the parent HGF molecule by abolishing the agonistic activity of the NK1 segment to bind to c-met.

This issue was further examined in experiments in which nitrated NK1 was evaluated directly for its c-met-binding activity by a sandwich ELISA-like assay (Fig. 4). Here, non-nitrated HGF (0.003 µM) demonstrated significantly high affinity to c-met and hence, served as a positive control (black bar in the left column). At the same molar concentration,

non-nitrated NK1 segment showed faint affinity to c-met (significantly higher than the negative control) than HGF (black bar in the mid column), consistent with the undetectable BrdU-incorporation activity of the 3.3×10^{-5} μM NK1, as shown in Fig. 3B. Importantly, at the higher molar concentration (0.015 μM), c-met binding activity was more clearly observed for the non-nitrated NK1 (black bar in the right column), with a significant decrease to a baseline level, depending on the nitration status determined in a peroxynitrite dose-dependent manner (in 1:100, 1:2000, and 1:4000 molar ratios relative to NK1) (Fig. 4, right column). These results strengthen the dysfunction model of nitrated NK1: in its central mechanism, it can not bind to the signaling receptor c-met and abolishes the HGF function to promote myogenic stem cell activation and subsequent proliferation.

3.4. *In vivo* nitration of HGF Y198

Finally, to clarify the physiological significance of the *in vitro* nitration/dysfunction of HGF and NK1 segment, a muscle group of rat lower hind-limb, EDL and TA muscles, from two aged-groups (2-month-old "young" and 12-month-old "retired/adult") was evaluated for the nitration level of ECM-bound HGF by direct fluorescence microscopy using anti-nitrated Y198 HGF monoclonal antibody. A pilot data set was shown in Fig. 5, in which extracellular HGF was clearly visualized by Alexa-probed anti-HGF α-chain antibody with a comparable fluorescence intensity between two aged-groups (panel A), while HGF nitration was more pronounced at the retired/adult stage (12-month-old) as revealed by the drastic increase in fluorescence intensity due to the binding of the Fluorescein-labeled anti-nitrated Y198 HGF antibody to the extracellular site (panel B). The result indicates that ECM-bound HGF undergoes nitration of Y198 *in vivo* and this progressive event is detectable at the early phase of aging (12 months of age).

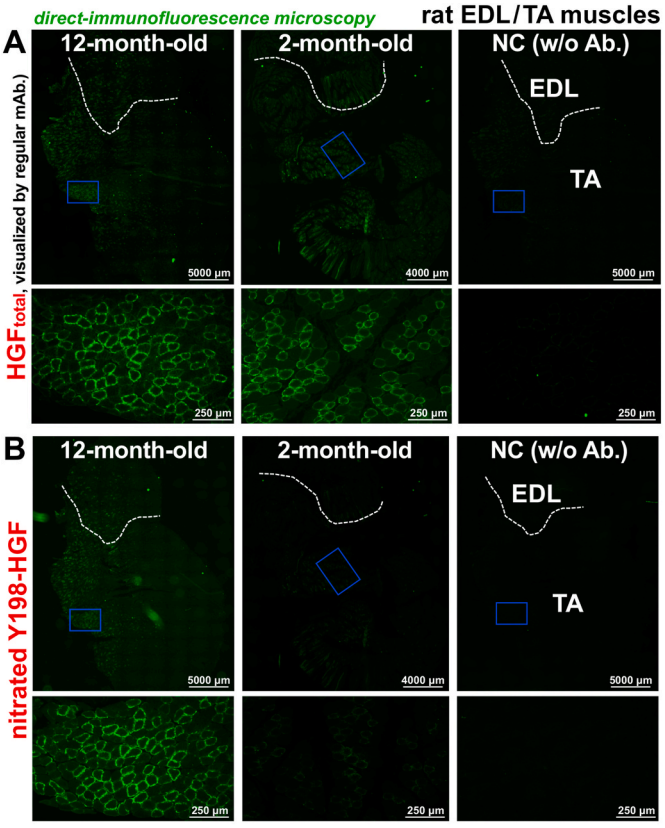

Fig. 5. *In vivo* nitration of extracellular HGF in muscle. A muscle group of EDL and TA muscles of lower hindlimb was collected from young (2-month-old) and retired/adult (12-month-old) rats; cryo-sections were examined for nitration of ECM-bound HGF by direct-immunofluorescence microscopy under the Tile-Scan program. (**A**) Visualization of ECM-bound HGF by Alexa Fluor 594-labeled anti-HGF monoclonal antibody; the fluorescent color was converted from the original red to green (pseudo color) for better visibility of fluorescent signals. (**B**) Serial cross-sections stained with Fluorescein 500-labeled anti-nitrated Y198 HGF monoclonal antibody. Each panel displays representative low-magnification views of whole muscle (first row) and magnified views of boxed areas (second row). NC, 12-month-old specimens without antibody treatment. The boundary between EDL and TA muscles is traced with a white dashed line.

4. Discussion

The list of tyrosine-nitrated proteins has lengthened through reports that proteins lose their biological activities upon nitration and that protein nitration has been detected in multiple organs including heart, brain, lung, liver, and colon [5–11]. In skeletal muscle tissue, nitration of myofibrillar proteins including α-actin, myosin light chain, tropomyosin, and α-actinin has been reported and supposed to impair the muscle contraction, although the detailed mechanisms of those dysfunctions are still unclear [50–53]. Since protein tyrosine-nitration may have a drastic biological impact on skeletal muscle performance, it is fascinating to clarify the possible correlation between muscle protein nitration and postnatal muscle growth and regeneration, especially if myogenic growth factors are also found to become nitrated. HGF is a dominant myogenic stem cell activator, required for entry to the cell cycle in postnatal muscle growth and regeneration; here we report that recombinant HGF undergoes tyrosine nitration in the α-chain under the physiological conditions and that this change disrupts the activation functionality of HGF/NK1 *in vitro*. The nitration potential of tryptophan residue(s) may not participate in this inhibitory mechanism, because nitrated tryptophan (6-nitrotryptophan) was not detected in recombinant HGF treated with peroxynitrite even at 1:4000 (molar ratio of HGF to peroxynitrite) under physiological conditions (pH 7.4 at 37°C for 30 min), by Western blotting of the nitrated HGF and a positive control (recombinant human nitrated SOD1 [54,55]) with monoclonal anti-nitrotryptophan antibody (data not shown). The involvement of oxidation of cysteine residues (thiol group of the side chain) [56,57] may be also excluded since all cysteine residues in the NK1 segment form disulfide bonds as shown by the crystal-structure analysis (see Fig. S3 panel B) [58], while oxidation of a methionine residue (Met-155) [59] might be a possible element in the inhibitory mechanism concerned here, awaiting further study using synthetic nitrated/non-nitrated NK1. Nonetheless, the physiological significance of *in vitro* HGF nitration was encouraged by direct-immunofluorescence microscopy of rat muscles from 2-month-old and 12-month-old groups, which clearly detected *in vivo* tyrosine nitration of extracellular HGF (Y198) more pronouncedly at 12-months old of the early aging-phase.

The functional significance of HGF nitration largely depends on chemical modification sites; we clarified that the nitration site(s) locates in NK1, a critical segment for the biological activity of HGF to bind to the signaling receptor c-met. While NK1 contains six tyrosine residues (Fig. S1 panel B), not all tyrosine residues within a protein are nitrated [2,45,60]. Several factors may be involved in tyrosine nitration selectivity: exposure of the tyrosine aromatic-ring to the protein surface may facilitate the initial attack by peroxynitrite while the probability for nitration of buried tyrosine residues is low [61]. The residues in the vicinity of a tyrosine residue are also critical for its possible nitration: a tyrosine close to a glutamate has a high tendency for nitration [62]. The crystal-structure analysis of the NK1 segment showed that the aromatic ring of Y198 may be exposed to the protein surface [37,63]; since Y198 is itself, adjacent to a glutamate residue (Fig. S1 panel A), Y198 was therefore considered as the best candidate for examining the impact of nitration in the present study.

This insight was supported by our Western blotting analyses that lead to a harmonious conclusion that Y198 may be the primary target for nitration in the NK1 segment. Since Y198 is essential for c-met binding

to generate the satellite cell activation signal [37,63] as supported by the 3D-structure analysis of a HGF-c-met complex [64] (see Fig. S3 panel A for better understanding) , it appears reasonable to propose that nitrated Y198 is the specific location of the nitration-induced dysfunction of HGF/NK1 that disrupts activation of quiescent satellite cells by triggering conformational changes that alter the function and activity of the HGF/NK1 protein. Nitration disturbs protein function by a variety of mechanisms, and the direct disabling of nitrated-protein binding with its cognate receptor may be one of those mechanisms [65,66]. Conversely, loss of protein function requires a chemical modification at specific critical tyrosine residues. The conformational changes induced by Y198 nitration may lead to defective NK1-c-met interaction, which underpins a dysfunctional mechanism of HGF nitration to abolish the signal transduction responsible for activation and the subsequent proliferation of satellite cells. This important issue awaits X-ray diffraction crystallographic study on the NK1 segment with and without nitration.

In conclusion, our findings of HGF nitration as a possible inhibitory mechanism for satellite cell activation and the identification of the HGF/NK1 tyrosine nitration site at Y198 suggest a relevant biomedical strategy could be designed to assist in preventing HGF nitration and its subsequent dysfunction, to attenuate or delay a variety of muscle pathologies and also to improve meat production and animal welfare.

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Appendix A. Supplementary data

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