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Association of neutrophil extracellular traps with the development of idiopathic osteonecrosis of the femoral head

Short title: NETs in idiopathic ONFH

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Abstract

Idiopathic osteonecrosis of the femoral head (ONFH) is defined as necrosis of osteocytes due to a non-traumatic ischemia of the femoral head. Iatrogenic glucocorticoid administration and habitual alcohol intake are regarded as risk factors. It has been suggested that glucocorticoid-induced activation of platelets contribute to the local blood flow disturbance of the femoral head. Both activated platelets and alcohol can induce neutrophil extracellular traps (NETs). To determine the association of NETs with the development of idiopathic ONFH, surgically resected femoral heads of patients with idiopathic ONFH and osteoarthritis (OA) were assessed for existence of NET-forming neutrophils by immunofluorescent staining. As a result, NET-forming neutrophils were present in small vessels surrounding the femoral head of patients with idiopathic ONFH but not OA. Moreover, Wistar-Kyoto rats were intravenously injected with NET-forming neutrophils or neutrophils without NET induction, and then the ischemic state of the tissue around the femoral head was evaluated by immunohistochemistry for hypoxia-inducible factor-1α (HIF-1α). NET-forming neutrophils circulated into the tissue around the femoral head, and HIF-1α expression in the tissue was higher compared to that of rats intravenously administered with neutrophils without NET induction. Furthermore, ischemic change of osteocytes was observed in the femoral head of rats given an intravenous injection of NET-forming neutrophils. The collective findings suggest that NETs are possibly associated with the development of idiopathic ONFH.

Introduction

Idiopathic osteonecrosis of the femoral head (ONFH) is defined as necrosis of osteocytes due to a non-traumatic ischemia of the femoral head.[1] It is a refractory disease that causes a collapse of the femoral head and a dysfunction of the hip joint, resulting in a decline in quality of life. Although the pathogenesis has not been revealed, it is well known that iatrogenic glucocorticoid administration and habitual alcohol intake are the risk factors for idiopathic ONFH.[2] A previous survey has demonstrated that, in patients with idiopathic ONFH, 51% were associated with glucocorticoid, 31% with alcohol, and 3% with both.[3]

Recent studies have suggested that, in addition to a certain genetic background, insufficient blood supply to the femoral head, influence of lipid metabolism, decrease in osteogenesis, and apoptosis of osteocytes—all these can be caused by glucocorticoid—are related to glucocorticoid-associated ONFH.[4] The contribution of platelet activation induced by glucocorticoid to the circulatory disorder of the femoral head has been pointed out.[5] In contrast, it has been shown that an excessive consumption of alcohol reduces the differentiation ability of osteoblasts.[6] As all patients who abuse alcohol do not develop ONFH, it is considered that a certain genetic factor is also involved in the susceptibility of alcohol-associated ONFH.[7]

Neutrophil extracellular trap (NET) is a web-like structure released from activated neutrophils, which consists of unraveled DNA accompanied by cytoplasmic antimicrobial proteins.[8] NETs can trap microorganisms by the web-like DNA, kill them by antimicrobial proteins, and consequently prevent the host from spreading the microbes. Although NETs play an essential role in innate immunity, an excessive formation of NETs can be a cause of microangiopathy.[9] Both activated platelets and alcohol can induce NETs.[10,11] Thus, we have hypothesized that if there are NET-forming

neutrophils—defined as neutrophils in the process of NET release—in small vessels surrounding the femoral head, these cells can be associated with the development of idiopathic ONFH.

In this study, we first examined the existence of NET-forming neutrophils in small vessels surrounding the femoral head of idiopathic ONFH and osteoarthritis (OA) as a disease control by immunofluorescent staining. As expected, NET-forming neutrophils were present in small vessels surrounding the femoral head of idiopathic ONFH but not of OA. Next, we attempted to inject NET-forming neutrophils intravenously into rats to determine whether NETs in small vessels surrounding the femoral head can induce ischemia of the tissue. It has been shown that NETs are degraded by serum factors, including DNase I,[12] and that serum DNase I activity is influenced by aging.[13] Thus, before conducting intravenous injection, we compared the serum ability of NET degradation between young adult (4-week-old) and senior aged (24-week-old) rats. Interestingly, serum NET degradation ability was significantly lower in senior aged rats than in young adult rats. Based on these findings, we injected NET-forming neutrophils intravenously into senior aged rats. This study demonstrates that intravenously injected NET-forming neutrophils circulated into the tissue around the femoral head and suggests that this event can induce ischemia of the femoral head.

Materials and Methods

Human subjects

The femoral heads of patients (50–87 years old), which were surgically resected with total hip arthroplasty from April 2018 to October 2019 at the Department of Orthopedic Surgery, Hokkaido University Hospital, were used in this study. These femoral heads were derived from 14 idiopathic ONFH patients (50–86 years old, 6 females) and 12 OA patients (52–87 years old, 7 females). The age and gender variance was not statistically different between idiopathic ONFH and OA patients (**Table 1**). Among the 14 idiopathic ONFH patients, 4 patients were associated with glucocorticoid medication, 2 patients with alcohol abuse, and 8 patients with both (**Table S1**). Underlying diseases that required glucocorticoid treatment included ulcerative colitis, bullous pemphigus, chronic inflammatory demyelinating polyneuropathy, pulmonary aspergillosis, systemic sclerosis, rheumatoid arthritis, drug-induced hypersensitivity syndrome, eosinophilic granulomatosis with polyangiitis, graft-versus-host disease after bone marrow transplantation, polymyositis, IgA nephritis, and rheumatic interstitial pneumonitis.

Written informed consent was obtained from all the patients. This study was approved by the Ethics Committee of Hokkaido University Hospital (Approval No. 018-0075).

Pathological diagnosis

The resected femoral heads were fixed with 10% formalin and then decalcified in 10% ethylenediaminetetraacetic acid (EDTA)-2Na solution. Several months later, the presence or absence of necrosis in the coronary section of the femoral head was determined macroscopically. Thereafter, macroscopic diagnosis was confirmed microscopically.

Immunofluorescent staining for NET-forming neutrophils

To determine the presence of NET-forming neutrophils in small vessels surrounding the femoral head, the connective tissue containing the synovial membrane appended to the femoral head was sampled before decalcification. The formalin-fixed connective tissue samples were then embedded in paraffin. These formalin-fixed paraffin-embedded (FFPE) tissues were sliced into 4 μm sections and then deparaffinized with xylene. The deparaffinized sections were autoclaved at 121℃ for 20 min with Tris-EDTA buffer (pH 9.0) to retrieve antigens and then soaked in Protein Block Serum-Free (Dako, Glostrup, Hovedstaden, Denmark) for 10 min to avoid the non-specific reaction of antibodies. Thereafter, the sections were made to react first with goat anti-human myeloperoxidase (MPO) antibody (1:200 dilution; R&D Systems, Minneapolis, MN, USA) overnight at 4℃ and next with rabbit anti-human citrullinated histone H3 (Cit-H3) antibody (1:100 dilution; abcam, Cambridge, MA, USA) as a NET marker[14] for 60 min at room temperature. The sections were then allowed to react with Alexa Fluor 488-conjugated donkey anti-goat IgG antibody (1:500 dilution; abcam) and Alexa Fluor 594-conjugated donkey anti-rabbit IgG antibody (1:500 dilution; abcam) for 60 min in the dark at room temperature. Finally, the sections were mounted with VECTASHIELD Mounting Medium with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) and observed under a fluorescence microscope.

Rats

Male Wistar Kyoto (WKY) rats (young adult, 4 weeks old; senior aged, 24 weeks old) were purchased from Sankyo Laboratory (Sapporo, Japan). Experiments using rats were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Hokkaido University (Permission No. 15-0034).

Measurement of NET degradation ability of rat serum

Peripheral blood neutrophils from healthy volunteers were seeded in the wells of four-well chamber slides (1×10^6 cells/ml). The cells were exposed to 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37°C to induce NETs. After washing with phosphate-buffered saline (PBS), the samples were incubated in 10% serum of young adult (4-week-old) WKY rats (n=5) and senior aged (24-week-old) WKY rats (n=5) for 30 min at 37°C. Thereafter, the samples were washed with PBS and then fixed with 4% paraformaldehyde for 15 min at room temperature. After washing with PBS, the remaining samples were mounted with the DAPI-containing solution. Photomicrographs (magnification, ×200) were taken randomly (six fields per well). The DAPI-positive area was measured using ImageJ software version 1.50i (https://imagej.nih.gov/ij/index.html; accessed on March 13, 2018), and the NET degradation rate (%) was calculated as described elsewhere.[15] The use of human peripheral blood was permitted by the Ethics Committee of the Faculty of Health Sciences, Hokkaido University (Permission No. 18-34).

Collection of neutrophils from the abdominal cavity of rats

WKY rats (4 weeks old) were given an intraperitoneal injection of 3% thioglycolate (Becton Dickinson, Franklin Lakes, NJ, USA) to induce peritonitis. After 48 h, inflammatory cells infiltrating into the peritoneum were collected by flushing the abdominal cavity with—and then re-suspending the washed-out cells in—PBS containing 1 mM EDTA. Neutrophils comprised more than 90% of the collected cells.

Induction of NETs and collection of NET-forming neutrophils

The collected cells were re-suspended in RPMI 1640 medium supplemented with 10%

fetal bovine serum and then seeded in 10 cm culture dishes (1×10^6 cells/ml). The cells were then exposed to 100 nM PMA for 4 h at 37ºC to induce NETs. After washing with PBS, the substance that remained on the dishes, which contained abundant NET-forming neutrophils, was collected using a cell scraper and then re-suspended in PBS.

Injection of NET-forming neutrophils into rats

The substance that contained abundant NET-forming neutrophils was injected into senior aged (24-week-old) WKY rats (Group 1, n=6) via the tail vein (substance derived from 2×10^7 cells/kg recipient weight) twice with 1-week interval. As controls, the cells collected from the abdominal cavity of rats with peritonitis were similarly injected into senior aged (24-week-old) WKY rats (Group 2, n=4; 2×10^7 cells/kg recipient weight) without NET induction. One week after the last injection, all rats were euthanized for histopathological evaluation.

Histopathological evaluation

The systemic organs and the connective tissue surrounding the femoral head were resected. The FFPE tissues were cut into 4 μm sections, deparaffinized with xylene, and then subjected to hematoxylin and eosin (HE) staining and elastica-van Gieson (EVG) staining. The sections of the connective tissue surrounding the femoral head were also subjected to immunofluorescent staining for NETs and immunohistochemistry (IHC) for hypoxia-inducible factor 1α (HIF-1α). IHC for HIF-1α was done as follows: FFPE sections were autoclaved at 121℃ for 10 min with Tris-EDTA buffer (pH 9.0) to retrieve antigens and then exposed to methanol supplemented with 3% hydrogen peroxide for 10 min to consume endogenous peroxidase in the tissue. After blocking the non-specific reaction of antibodies with 10% goat serum, the sections were made to

react with mouse anti-human HIF-1α antibody (1:800 dilution; abcam), which can cross-react with rat HIF-1α, for 60 min at room temperature. After washing out unbound antibodies with PBS, the sections were next allowed to react with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (1:50 dilution; abcam) for 60 min at room temperature. HRP activity was developed with diaminobenzidine.

Statistical analysis

χ^2 test was applied for the comparison of the difference in frequency between two groups. Mann-Whitney *U*-test was applied for the comparison of values between two nonparametric groups. $p<0.05$ was regarded as statistically significant.

Results

Macroscopic and microscopic findings of the coronary section of the femoral head

The resected femoral heads were fixed with 10% formalin and then decalcified in 10% EDTA-2Na solution. Several months later, the presence or absence of necrosis in the coronary section of the femoral head was determined macroscopically. Overt necrosis was observed in the coronary section of the femoral heads derived from idiopathic ONFH patients (**Figure 1A**). Necrosis was not apparent in the coronary section of the femoral heads derived from OA patients (**Figure 1C**). Afterward, these macroscopic findings were confirmed microscopically (**Figures 1B** and **1D**).

NET-forming neutrophils in small vessels surrounding the femoral head

The connective tissue containing the synovial membrane appended to the femoral head (**Figure 2A**) was sampled before decalcifying the bone and subjected to HE staining and immunofluorescent staining for NET-forming neutrophils. In all 14 cases of idiopathic ONFH, NET-forming neutrophils identified by a co-localization of Cit-H3 (NET marker[14]), MPO, and DAPI (DNA marker) were found in small vessels surrounding the femoral head (**Figure 2B**), whereas no thrombus was found in the HE specimens. In contrast, MPO^+ cells but not NET-forming neutrophils were observed in small vessels surrounding the femoral head in all 12 cases of OA (**Figure 2c**). There was a statistically significant difference in the presence of NET-forming neutrophils in small vessels surrounding the femoral head between idiopathic ONFH and OA (**Table 2**; χ^2 test, $p=3.4\times10^{-7}$).

Serum NET degradation ability was lower in senior aged rats than in young adult rats

We compared the serum NET degradation ability between young adult (4-week-old) and

senior aged (24-week-old) WKY rats. In brief, NETs induced on human peripheral blood neutrophils by PMA were exposed to 10% rat serum for 30 min at 37℃, and then residual NETs were quantified by image analysis. As a result, the serum NET degradation ability of senior aged rats was found to be significantly lower than that of young adult rats (**Figures 3A–3E**).

Circulation of NET-forming neutrophils into the tissue around the femoral head of rats intravenously injected with NET-forming neutrophils

According to the results of the serum NET degradation assay, NET-forming neutrophils were injected into senior aged (24-week-old) WKY rats (Group 1, n=6) via the tail vein (substance derived from 2×10^7 cells/kg recipient weight) twice with 1-week interval. For this purpose, cells collected from the abdominal cavity of WKY rats with peritonitis were stimulated by PMA to induce NETs in culture dishes. After removal of PMA by washing with PBS, the residual substance that contained abundant NET-forming neutrophils was injected into rats. As controls, the cells collected from the abdominal cavity of rats with peritonitis were similarly injected into senior aged (24-week-old) WKY rats (Group 2, n=4; 2×10^7 cells/kg recipient weight) without NET induction. In Group 1, NET-forming neutrophils were found in small vessels at either the left or right connective tissue around the femoral head in all 6 rats (8/12 legs; **Figure 4A**). In Group 2, MPO^+ cells but not NET-forming neutrophils were observed in small vessels surrounding the femoral head (0/8 legs; **Figure 4B**). There was a statistically significant difference in the presence of NET-forming neutrophils in small vessels surrounding the femoral head between Groups 1 and 2 (**Table 3**; χ^2 test, p=0.0029).

Ischemic change of the femoral head of rats intravenously injected with NET-forming neutrophils

To determine the ischemic state of the femoral head of WKY rats intravenously injected with NET-forming neutrophils, we examined the expression of HIF-1α in the connective tissue appended to the femoral head. Results demonstrated that HIF-1α expression in vascular smooth muscle cells in the connective tissue around the femoral head of Group 1 was higher compared to that of Group 2 (**Figures 5A** and **5B**). Correspondingly, ischemic change of osteocytes in the femoral head represented by a decrease in nuclear staining for hematoxylin was observed in two legs of two rats in Group 1 (2/12 legs; **Figures 5C** and **5E**), although no thrombus was found in the connective tissue around the femoral head. Ischemic change of osteocytes in the femoral head was not apparent in Group 2 (0/8 legs; **Figure 5D** and **5F**). Although there was no statistically significant difference between Groups 1 and 2 (**Table 4**; χ^2 test, p=0.22), the collective findings suggested the association of NET-forming neutrophils in small vessels surrounding the femoral head with ischemia of the femoral head.

Discussion

In this study, we first demonstrated the presence of NET-forming neutrophils in small vessels surrounding the femoral head of patients with idiopathic ONFH but not OA. It has been considered that ONFH is developed consequently to a compromised subchondral microcirculation.[16] Because of the limitation of methodology—immunofluorescent staining for NETs prefers tissue sections without decalcification, we assessed small vessels in the connective tissue surrounding the femoral head instead of the subchondral small vessels in the bone. Results indicate the association of idiopathic ONFH and the presence of NET-forming neutrophils in small vessels near to the lesion. However, it is elusive whether NET-forming neutrophils are involved in the development of idiopathic ONFH or are present there as a result of the disease. Therefore, we next planned to conduct animal experiments.

Diverse animal models of idiopathic ONFH, including glucocorticoid-induced ONFH in rats, mice, rabbits, chickens, pigs, and emus (reviewed by Xu et al.[17]) and alcohol-induced ONFH in rats,[18] goats,[19] and canine,[20] have been established. Although these models mimic the clinical characteristics of idiopathic ONFH, we contrived a simple induction model to determine the direct contribution of NET-forming neutrophils to the development of ONFH. For this purpose, we employed inbred wild-type WKY rats that are often used for vascular research focusing on vasodynamics[21] and vasculitides.[22]

NET-forming neutrophils intravenously injected into WKY rats circulated into the tissue around the femoral head and the expression of HIF-1α in the tissue was higher compared to the control tissue. Correspondingly, ischemic change of osteocytes in the femoral head represented by a decrease in nuclear staining for hematoxylin was observed in some rats given an intravenous injection of NET-forming neutrophils. It has

been demonstrated that the expression of HIF-1α is induced by tissue hypoxia.[23] Based on these findings, we consider that NETs are possibly involved in the local blood flow disturbance and ischemia of the femoral head. However, factors other than NETs could be also involved in the pathogenesis of ONFH because an apparent necrosis of osteocytes that resulted in the destruction of the femoral head was not observed in the rats.

It should be noted that overt thrombi have not been found in the connective tissue around the femoral head of either patients with ONFH or the rats given an intravenous injection of NET-forming neutrophils. Although NETs link thrombosis,[24] mechanisms other than thrombogenesis, e.g.; spasm of small vessels with NET-mediated injury of endothelial cells, may be involved in the tissue ischemia. Further studies are needed to determine how NET-forming neutrophils in small vessels contribute to tissue ischemia.

NET-forming neutrophils do not seem to be recruited into the tissue around the femoral head site-specifically, because the deposition of NETs in the small vessel wall has been also observed in other sites of connective tissues in the rats (**Figure S1**). These findings suggest that the intravenously injected NET-forming neutrophils are circulating in the body of rats. However, we do not deny the possibility that a certain anatomical factor is associated with the local blood flow disturbance of the femoral head.[25]

In this study, we regarded cells exhibiting a co-localization of Cit-H3, MPO, and DAPI as NET-forming neutrophils. However, these cells may include extracellular trap-forming monocytes, as monocytes—which also contain MPO—have been shown to form extracellular traps similarly to neutrophils.[26] Additional studies are needed to identify Cit-H3$^+$, MPO$^+$, and DAPI$^+$ cells as NET-forming neutrophils.

The amount of NETs *in vivo* is estimated as a balance of generation and degradation. The generation of NETs is physiologically induced by infectious stimuli,

such as lipopolysaccharides.[8] Under pathological conditions, diverse factors that activate neutrophils, including inflammatory cytokines and autoantibodies, can induce NETs.[27] These NET-inducible factors are generally associated with autoimmune diseases, which are the targets of glucocorticoid treatment. In addition, it has been shown that the number of low-density granulocytes, which are apt to generate NETs, is increased in patients with autoimmune diseases, including systemic lupus erythematosus (SLE).[28] Therefore, it is considered that patients who developed glucocorticoid-associated ONFH have been liable to generate NETs due to underlying diseases. Further studies are needed to clarify how glucocorticoid enhances NET formation on neutrophils of patients with diverse autoimmune diseases. Bukong *et al*. have recently demonstrated that alcohol can induce NETs *in vitro*.[11] Thus, it is also considered that patients who developed alcohol-associated ONFH have been liable to generate NETs.

The two major mechanisms of NET degradation are digestion by serum DNase I[12] and efferocytosis by macrophages.[29] The low ability of NET degradation in the sera of patients with autoimmune diseases, including SLE[12] and microscopic polyangiitis,[30] has been demonstrated. Moreover, the impairment of efferocytosis in SLE has been shown.[31] Interestingly, alcohol binges induced a reduction of efferocytosis by macrophages in mice.[11] The determination of NET degradation disorder in patients with idiopathic ONFH is a critical subject in future studies.

Conclusions

Idiopathic ONFH develops in patients with a predisposing genetic background who have been exposed to causative environmental factors. This study suggests the association of NETs with the development of idiopathic ONFH and promotes the understanding of the pathophysiology.

Acknowledgements

T.S., S.M., D.N., U.T., and A.I. conceived the study, designed the experiments, and analyzed data. M.N., T.S., M.Y., Y.H., Y.N., D.T., T.A., Y.N., S.M., and A.I. performed the experiments. M.N., T.S., S.M., D.N., S.T., U.T., N.I., and A.I. discussed results. All authors read and approved the final version of the manuscript.

Supplemental Data

Table S1. Idiopathic ONFH patients enrolled in this study

Figure S1. Deposition of NETs into the small vessel wall in connective tissue of WKY rats given intravenous injection of NET-forming neutrophils

(**A**) HE staining. Bar, 100 μm. (**B**) EVG staining. Bar, 100 μm. (**C**) Immunofluorescent staining of NETs. Red, Cit-H3; Blue, DNA. The white arrowhead indicates the deposition of NETs. Bar, 50 μm.

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Table 1. Patients enrolled in this study

Demographics	Idiopathic ONFH (*n*=14)	OA (*n*=12)	*p*
Age (mean ± SD)	61.8 ± 9.3	68.1 ± 9.4	0.11
Sex (F/M)	6/8	7/5	0.23

Table 2. Existence of NET-forming neutrophils in small vessels surrounding the femoral head

Patients	NET-forming neutrophils		*p*
	Absent	Present	
Idiopathic ONFH	0	14	3.4×10^{-7}
OA	12	0	

Table 3. Existence of NET-forming neutrophils in small vessels surrounding the femoral head

Rat group	NET-forming neutrophils		*p*
	Absent	Present	
Legs of Group 1	4	8	0.0029
Legs of Group 2	8	0	

Table 4. Ischemic change of osteocytes in the femoral head

Rat group	Ischemic change of osteocytes		*p*
	Absent	Present	
Legs of Group 1	10	2	0.22
Legs of Group 2	8	0	

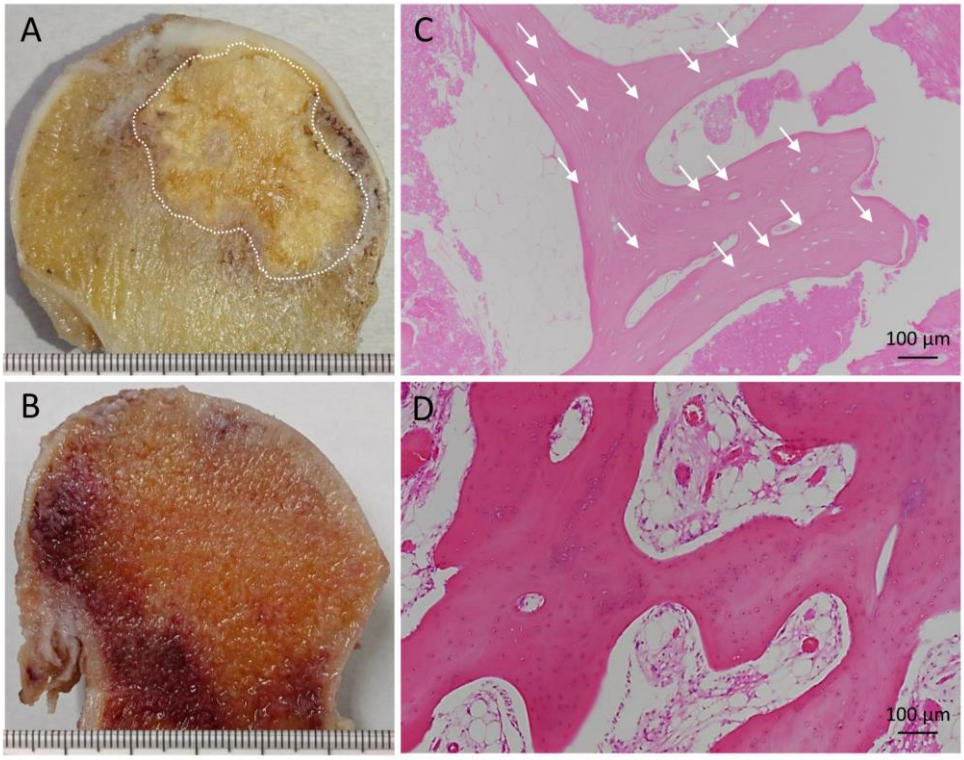


Figure 1. Macroscopic and microscopic findings of the coronary section of the femoral head

Representative macroscopic (**A** and **C**) and microscopic (**B** and **D**) findings of the coronary section of the femoral head of idiopathic ONFH (**A** and **B**) and OA (**C** and **D**). The area enclosed by the broken line exhibits necrosis. White arrows indicate osteocytes with disappeared nuclei. Bar, 100 μm.

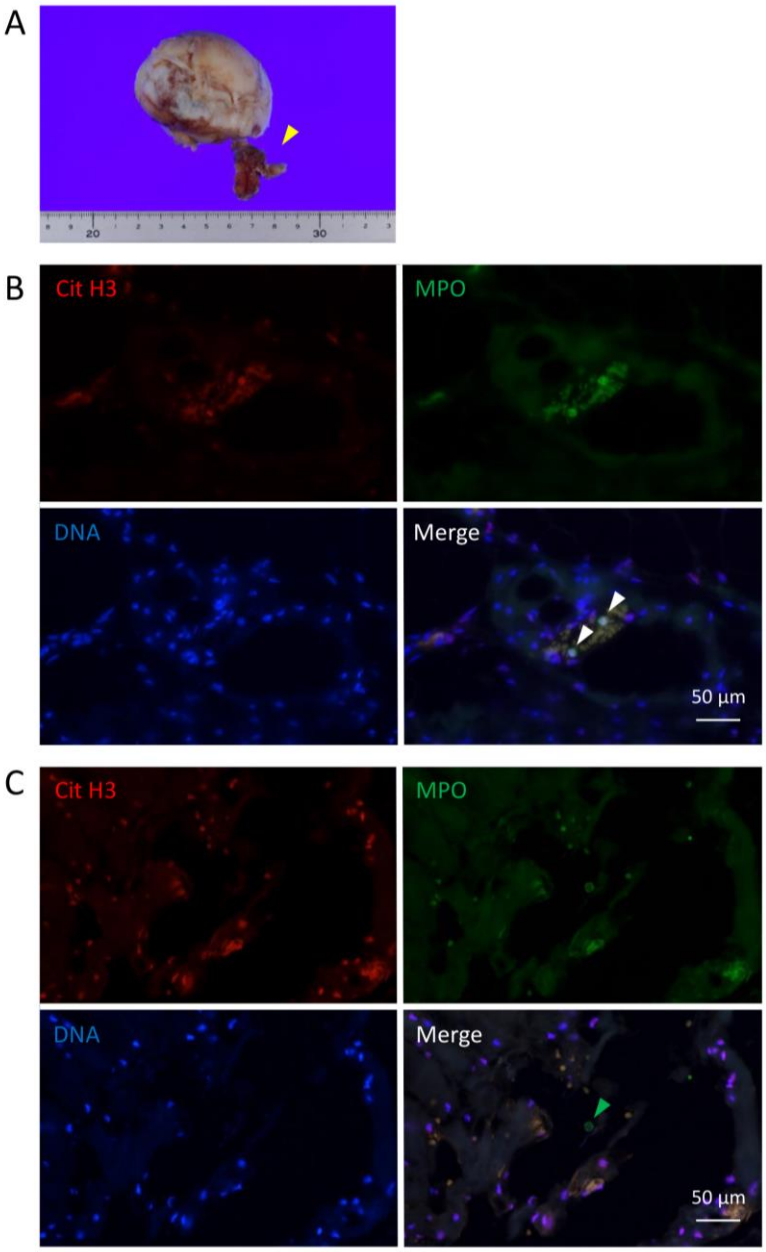


Figure 2. NET-forming neutrophils in small vessels surrounding the femoral head
(**A**) Connective tissue containing the synovial membrane appended to the femoral head (yellow arrowhead), which was subjected to immunofluorescent staining. (**B**) Representative findings of NET-forming neutrophils (white arrowheads) in the tissue around the femoral head of idiopathic ONFH. (**C**) Representative findings of the tissue around the femoral head of OA. An MPO^{+} cell (green arrowhead) but not a NET-forming neutrophil was observed. Bar, 50 µm.

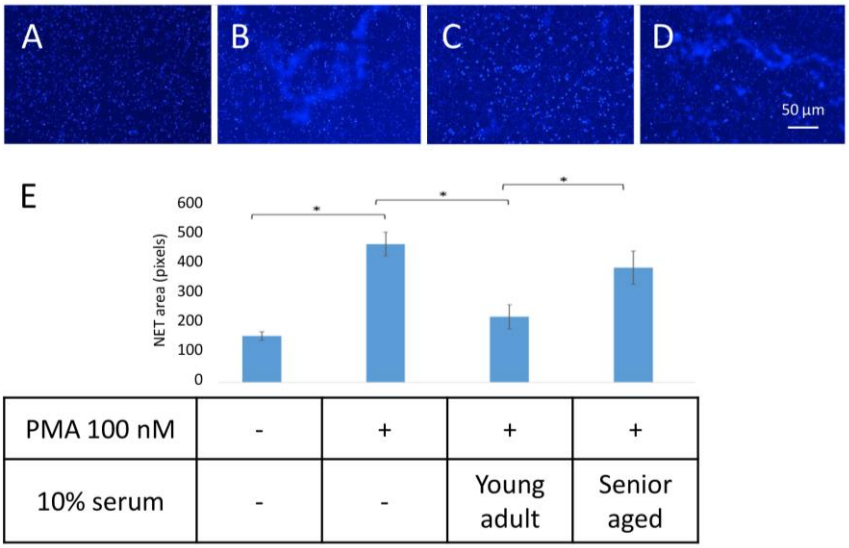


Figure 3. Serum NET degradation assay

Representative microphotographs: (**A**) 100 nM PMA (-), 10% serum (-); (**B**) 100 nM PMA (+), 10% serum (-); (**C**) 100 nM PMA (+), 10% serum of young adult (4-week-old) rats; and (**D**) 100 nM PMA (+), 10% serum of senior aged (24-week-old) rats. Bar, 50 µm. (**E**) Area of NETs determined using ImageJ software. Data are presented as mean ± standard deviation. *p<0.05.

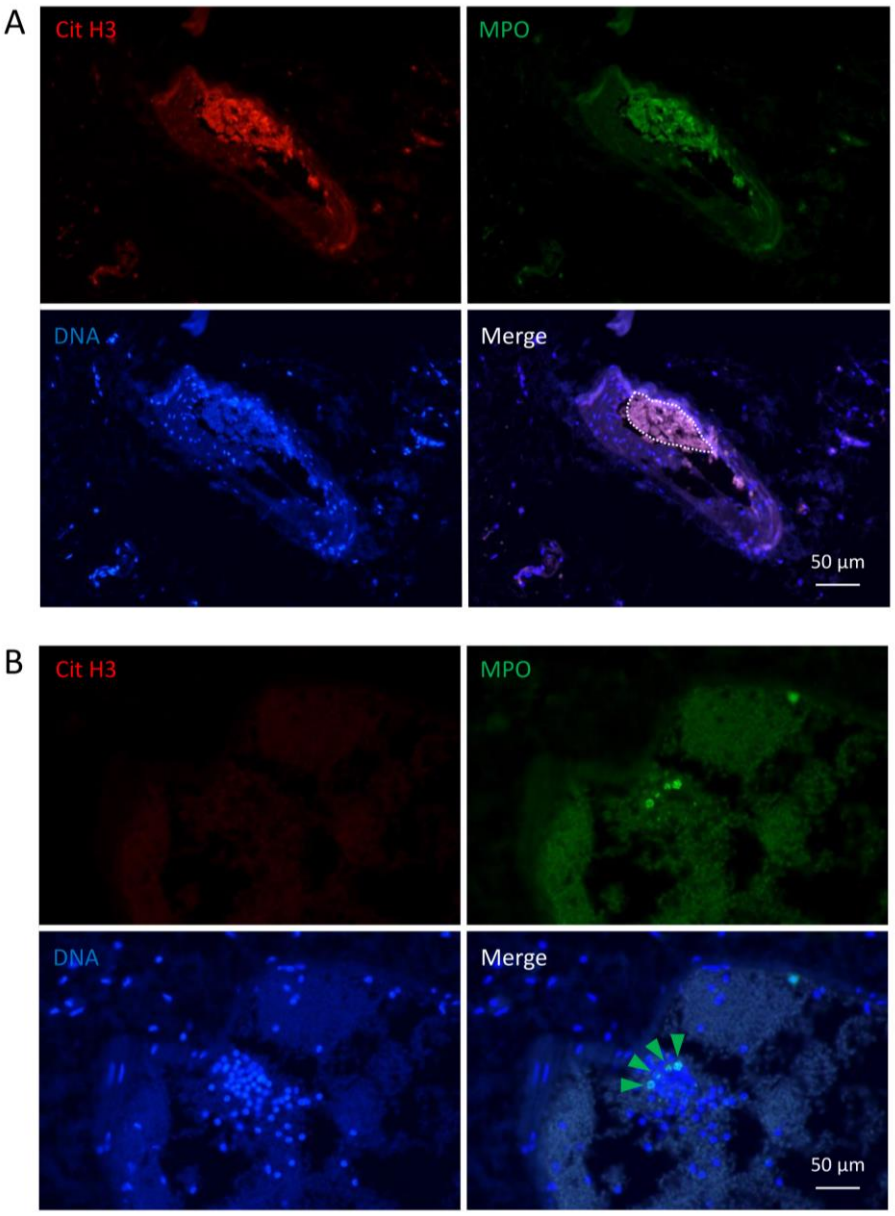


Figure 4. Circulation of NET-forming neutrophils into the tissue around the femoral head of WKY rats given intravenous injection of NET-forming neutrophils

(**A**) Representative findings of NET-forming neutrophils in the tissue around the femoral head of WKY rats given intravenous injection of NET-forming neutrophils (Group 1). A cluster of NET-forming neutrophils is enclosed by the broken line. (**B**) Representative findings of WKY rats given intravenous injection of neutrophils without NET induction (Group 2). MPO^+ cells (green arrowheads) but not NET-forming neutrophils were observed. Bar, 50 µm.

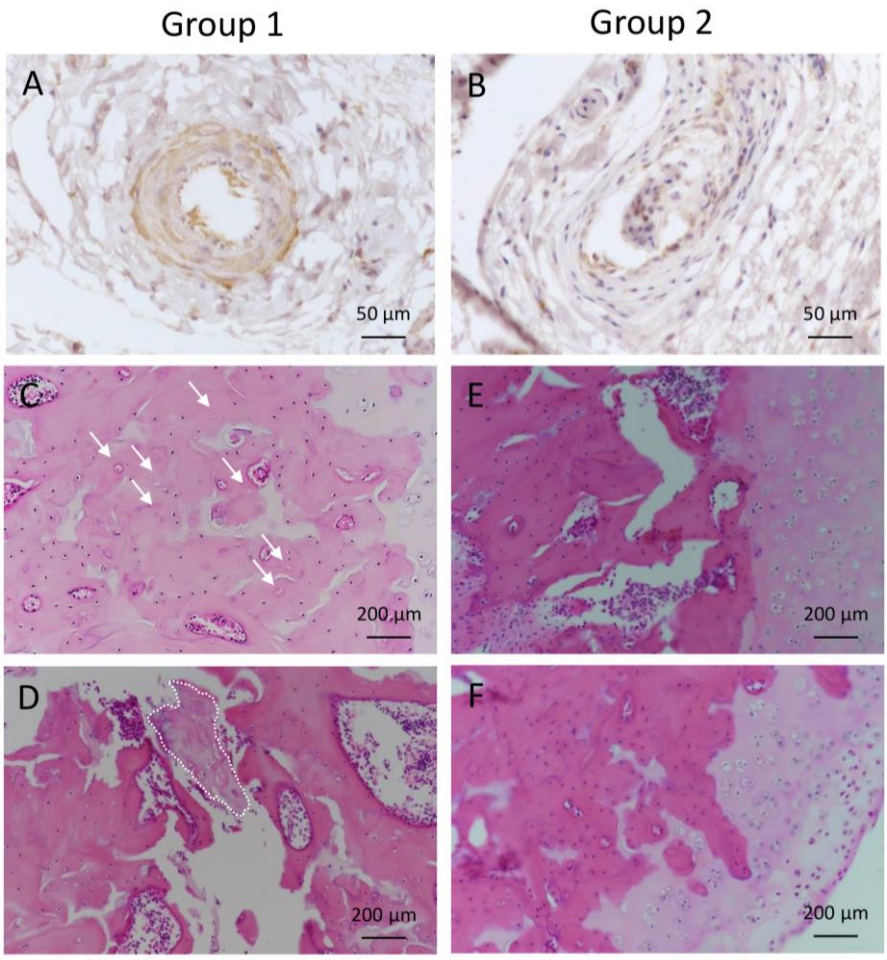


Figure 5. Ischemic state of the femoral head

The femoral heads and surrounding tissue of WKY rats given intravenous injection of NET-forming neutrophils (Group 1; **A**, **C**, and **E**) and WKY rats given intravenous injection of neutrophils without NET induction (Group 2; **B**, **D**, and **F**) were subjected to IHC for HIF-1α (**A** and **B**; Bar, 50 µm) and HE staining (**C**–**F**; Bar, 100 µm). Representative findings are shown. White arrows indicate osteocytes with disappeared nuclei. The area enclosed by the broken line exhibits a necrotic lesion.

Figure S1

