Nasal polyp fibroblasts (NPFs)-derived exosomes are important for the release of vascular endothelial growth factor from cocultured eosinophils and NPFs

ABSTRACT

Objective: Significant eosinophil infiltration and tissue remodeling are common characteristics of conditions associated with chronic airway inflammation, such as chronic rhinosinusitis with nasal polyp and bronchial asthma. This study was designed to elucidate the role of eosinophil-fibroblast interactions in tissue remodeling during chronic airway inflammation.

Methods: Peripheral blood eosinophils or EoL-1 eosinophilic leukemia cells were cocultured with nasal polyp fibroblasts (NPFs). Coculture-induced release of exosomes, major components of extracellular vesicles (EVs), and a profibrotic cytokine, vascular endothelial growth factor (VEGF), were evaluated by enzyme-linked immunosorbent assay.

Results: Eosinophil-NPF interactions stimulated the release of exosomes and VEGF into culture supernatants. Coculture-induced release of exosomes was stimulated earlier than VEGF release, at 3 h of incubation. The average size of the EVs released by NPFs was 133 ± 3.6 nm. NPF-derived EVs (exosome concentration: 25 pg/mL) significantly stimulated VEGF release from EoL-1 cells. Pretreatment of NPFs with exosome inhibitor, GW4869 or DMA attenuated the release of exosomes and VEGF from cocultured EoL-1 cells and NPFs.

Conclusion: The results of this study indicate that eosinophil-fibroblast interactions are important in the pathophysiology of tissue remodeling in eosinophil-predominant airway inflammation and that NPF-derived exosomes play a crucial role in the release of VEGF.

Key words: nasal polyps, eosinophils, fibroblasts, coculture, exosome, vascular endothelial growth factor

INTRODUCTION

Significant eosinophil infiltration and tissue remodeling in the airway epithelium are common characteristics of chronic inflammation in nasal polyposis and bronchial asthma(1, 2). Morphologic changes associated with tissue remodeling include epithelial thickening, subepithelial fibrosis, goblet cell hyperplasia, increased vascularity, polyp formation (in nasal polyposis), and increased smooth muscle mass (in bronchial asthma)(1, 3). Vascular endothelial growth factor (VEGF) is a profibrotic cytokine that contributes to tissue remodeling by increasing vascular permeability and leukocyte infiltration, by stimulating the proliferation of vascular endothelial cells, myocytes, and goblet cells, and by increasing the deposition of extracellular matrix(4, 5). Overexpression of VEGF and its receptors, VEGFR-1 and VEGFR-2, has been reported in nasal polyps (NPs) from patients with chronic rhinosinusitis with nasal polyp (CRSwNP)(6, 7).

Eosinophils, the principal effector cells in nasal polyposis and bronchial asthma, exert proinflammatory and destructive effects via the production of inflammatory mediators such as cytokines, chemokines, lipid mediators, reactive oxygen species, and toxic granular proteins(8). Epithelial cells also play an important role in airway inflammation by releasing a variety of inflammatory mediators, and epithelial cell-eosinophil interactions are crucial in the initiation, development, and resolution of these inflammatory processes(9, 10). It has been reported that the coculture of bronchial epithelial cells and eosinophils stimulates the production of cysteinyl leukotrienes and chemokines(11, 12). We previously reported that coculture of NCI-H292 human airway epithelial cells and EoL-1 eosinophilic leukemia cells or peripheral blood eosinophils stimulates the secretion of VEGF, platelet-derived growth factor (PDGF), interleukin (IL)-8, and MUC5AC mucin into the culture supernatant(13).

Fibroblasts are the major producers of extracellular matrix proteins in the airway, including collagens, fibronectin, and proteoglycans, and increased deposition of collagen and

fibronectin in NPs has been reported(14). Fibroblasts also release inflammatory mediators that contribute to tissue remodeling, such as growth factors, cytokines, and chemokines. We previously reported that thrombin and activated coagulation factor X stimulate the release of transforming growth factor-beta 1, fibronectin, eotaxin-1, IL-6, and IL-8 from nasal polyp fibroblasts (NPFs)(15). However, little is known about the role of eosinophil-fibroblast interactions in airway inflammation.

Extracellular vesicles (EVs) are membrane-contained vesicles released from almost all types of cells, and can be broadly classified into exosomes, microparticles, and apoptotic bodies. Recent research indicates that exosomes are potent mediators of intercellular communication(16). Exosomes, major components of EVs, are nanoscale (30-150 nm) vesicles released into almost all body fluids, including serum, urine, cerebrospinal fluids, joint fluids, and nasal mucus(17). Exosomes are formed within multivesicular bodies (MVBs) and are released following the fusion of MVB with the cell membrane. Exosomes containing proteins, lipids, DNA, mRNA, microRNA, and other bioactive substances, may be involved in eosinophil-fibroblast interactions. To elucidate the role of eosinophil-fibroblast interactions in tissue remodeling associated with chronic airway inflammation, the present study evaluated the release of exosomes and VEGF in coculture of eosinophils and NPFs. The effect of NPF-derived exosomes on the release of VEGF from EoL-1 cells was also examined.

MATERIALS AND METHODS

Cell culture

NPs were obtained during nasal surgery from three patients with eosinophilic CRSwNP(18). All three patients were comorbid with bronchial asthma, and two patients had aspirin-induced asthma. JESREC scores(18) of all three patients were 17, and peripheral eosinophil rates were 11.3%, 11.8 %, and 12.3%, respectively. Informed consent was obtained from all subjects before sampling. The clinical protocol was approved by the Shiga University of Medical Science Institutional Review Board for Clinical Investigation (R2019-149). Tissue specimens were cut into small pieces. NPFs were expanded in 75-cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM, WAKO, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 100 μg/mL penicillin, and 100 μg/mL streptomycin in a humidified incubator at 37°C with 5% CO_2. NPFs were passaged weekly by means of trypsin-EDTA . Once confluent, NPFs were subcultured and seeded into 24-well tissue culture plates. After incubation of the confluent cells for 18 h in DMEM without supplements, NPFs were co-cultured with peripheral blood eosinophils (100,000/mL) or EoL-1 human eosinophilic leukemia cells (1,000,000/mL) for an additional 24 h in DMEM without supplements. The supernatants were collected, centrifuged for 5 min at 2,000 × g to remove cells, and stored at −20°C until use.

GW4869 (Cayman Chemical, Ann Arbor, MI, USA), a neutral sphingomyelinase 2 inhibitor known to inhibit ceramide biosynthesis and reduce the number of exosomes released into culture medium(19), and 5-(N, N-dimethyl)-Amiloride hydrochloride (DMA) (Cayman Chemical) known to regulate intercellular Ca^{2+} and reduce exosome secretion(20) were used to determine whether exosomes are involved in VEGF production in cocultured cells. GW4869 and DMA were dissolved in dimethyl sulfoxide (DMSO) and the same concentration of DMSO was used as a control sample. NPFs were incubated with or without

GW4869 or DMA for 18 h in DMEM without supplements, washed, and then cocultured with EoL-1 cells for an additional 3, 6, or 24 h in DMEM without supplements. The supernatants were collected, centrifuged, and stored at −20°C until use. The concentration of exosome in the cell supernatants was determined using a CD9/CD63 enzyme-linked immunosorbent assay (ELISA) kit (COSMO BIO, Tokyo, Japan). The concentration of VEGF was determined using an immunoassay kit (R&D Systems, Minneapolis, MN, USA).

Eosinophil isolation

Human peripheral eosinophils were obtained from heparinized blood collected from healthy volunteers. Eosinophil isolation was performed as described previously using a MACS system (BD Biosciences, San Jose, CA, USA)(21). Randolph's staining indicated that eosinophil purity was >98%. EoL-1 cells were provided by the RIKEN BRC (Tsukuba, Japan) through the National Bio-Resource Project of MEXT, Japan. Eosinophils and EoL-1 cells were suspended in RPMI-1640 medium supplemented with 10% FBS, 100 μg/mL penicillin, and 100 μg/mL streptomycin.

Extracellular vesicles (EVs) isolation

NPFs were plated in 10 cm-diameter dishes in DMEM supplemented with 10% FBS. After reaching confluence, the cells were cultured in 10 mL of DMEM supplemented with 10% exosome-depleted FBS (Exo-FBS; System Biosciences, Palo Alto, CA, USA) for 7 days. The supernatants were collected and centrifuged for 5 min at 1,500 × g to remove cells, centrifuged for 5 min at 14,000 × g to remove debris, and further centrifuged for 60 min at 100,000 × g (Optima TL ultracentrifuge, Beckman Coulter, Brea, CA, USA). The purified EVs were resuspended in 500 μL phosphate-buffered saline, and the concentration of exosome was determined using a CD9/CD63 ELISA kit (COSMO BIO).

Nanoparticle tracking analysis (NTA)

EVs isolated from NPFs were analyzed using a Nanosight LM10 system (Nanosight Ltd., Novato, CA, USA). Nanoparticles were illuminated by the laser, and their movement under Brownian motion was captured by video for 60 s. The video was subjected to NTA using the Nanosight particle tracking software to determine the nanoparticle concentration and size distribution.

Transmission electron microscopy (TEM)

EVs solution was applied to copper grids, followed by negative staining with 2% uranyl acetate. The grids were then washed, dried, and viewed using a transmission electron microscope (Hitachi, Tokyo, Japan).

Statistical analyses

All data are expressed as the mean and standard deviation (SD). The statistical significance of differences was determined by one-way ANOVA or unpaired t-test. When one-way ANOVA indicated a significant difference, Tukey's honestly significant difference test or Games-Howell test was then used to assess the difference between groups. All analysis was performed using SPSS statistics software (IBM, Armonk, NY, USA). P values < 0.05 were considered indicative of a significant difference.

RESULTS

Coculture-induced release of exosomes and VEGF

Unstimulated NPFs released exosomes and VEGF, although the amount of VEGF release is different depending on the cultured NPFs. Peripheral blood eosinophils (100,000/mL) exhibited almost no release of exosomes and VEGF. Coculture of peripheral blood eosinophils and NPFs for 24 h stimulated the release of exosomes and VEGF (Fig. 1A and 1B). Unstimulated EoL-1 cells (1,000,000/mL) released VEGF but few exosomes. Coculture of EoL-1 cells and NPFs stimulated the release of exosomes and VEGF (Fig. 1C and 1D).

The pattern of exosome and VEGF release over time was examined at 3, 6, 12, and 24 h of incubation. Coculture-induced release of exosomes and VEGF was stimulated at 3 h of incubation. Coculture-induced exosome release continued to decrease for up to 24 h of incubation, although coculture-induced VEGF release continued to increase for up to 24 h of incubation (Fig. 2A and 2B). The peak of exosome release seemed to be earlier than the peak of VEGF release.

NPF-derived extracellular vesicles (EVs)

NTA using Nanosight was employed to determine the size and concentration of NPF-derived EVs. The average size of the EVs was 133 ± 3.6 nm, and the average count was 6.61 ± 0.05 × 10^8 particles/mL (Fig. 3A). The concentration of exosome was 510.8 pg/mL. The morphology of NPF-derived EVs as determined by TEM is shown in Figure 3B.

Effect of NPF-derived EVs on VEGF release from EoL-1 cells

NPF-derived EVs isolated by ultracentrifugation (exosome concentration: 25 pg/mL), and cultured NPFs-derived EVs alone did not produce VEGF for 24 hours. NPF-derived EVs

were incubated with EoL-1 cells for 20 h, resulting in significant stimulation of VEGF release from EoL-1 cells (Fig. 3C). NPF-derived EVs did not stimulate VEGF release from NPFs or from normal peripheral blood eosinophils (data not shown).

Effect of GW4869 and DMA, exosome inhibitors on coculture-induced release of exosomes and VEGF

Pretreatment of NPFs for 18 h with GW4869 (10 μM), an inhibitor of exosome production, attenuated the release of exosomes (Fig. 4A) and VEGF (Fig. 4B) at 24 h from cocultured EoL-1 cells and NPFs.

Pretreatment of NPFs for 18 h with DMA, an inhibitor of exosome secretion, attenuated the release of exosomes (Fig. 4C) and VEGF (Fig.4D) at 3, 6, and 24 h from cocultured EoL-1 cells and NPFs.

DISCUSSION

Significant eosinophil infiltration and tissue remodeling are characteristics of conditions associated with chronic airway inflammation, such as nasal polyposis and bronchial asthma(1, 3). The profibrotic cytokine VEGF is an important mediator of the cellular biological processes that lead to tissue remodeling. In the present study, interactions between eosinophils and NPFs induced the release of VEGF in cocultured cells. Overexpression of VEGF and its receptors is commonly reported in the upper and lower airways of patients with CRSwNP and bronchial asthma(6, 7, 22). In NPs, VEGF is expressed in epithelial cells, fibroblasts, vascular endothelial cells, and inflammatory cells, including eosinophils and macrophages(7). Eosinophils produce a variety of inflammatory mediators, including VEGF, and may play an important role in tissue remodeling during eosinophil-predominant chronic airway inflammation(8). Increased number of infiltrating eosinophils in NPs was shown to be associated with a higher risk of NP recurrence in patients with CRSwNP (23). The protein concentration and mRNA expression of VEGF were increased in human eosinophilic NP tissues, compared with non-eosinophilic NP tissues(24). We previously reported that coculture of NCI-H292 human airway epithelial cells and blood eosinophils or EoL-1 cells induce the release of VEGF, PDGF, IL-8, and MUC5AC mucin into the culture supernatant(13). This is the first report focusing on the role of eosinophil-fibroblast interactions in enhancing the release of a profibrotic cytokine involved in airway tissue remodeling.

In the present study, eosinophil-fibroblast interactions induced the release of exosomes and VEGF from cocultured blood eosinophils or EoL-1 cells and NPFs. Coculture-induced release of exosomes was stimulated earlier than induction of VEGF release, at 3 h of incubation. Pretreatment of NPFs with exosome inhibitor, GW4869 or DMA attenuated the coculture-induced release of exosomes and VEGF. These results indicate that NPF-derived

exosomes play a crucial role in VEGF release induced by eosinophil-fibroblast interactions. Exosomes are 30-150 nm EVs secreted by various somatic cells and have been detected in multiple body fluids, including nasal mucus, nasal lavage fluid (NLF), and bronchoalveolar lavage fluids (BALFs)(16). Exosomes contain proteins, lipids, mRNA, microRNA, and other bioactive substances specific to their cellular origin and the pathophysiologic state of the cells. Exosomes may provide various stimuli to the recipient cells, and in the present study, NPF-derived exosomes may subsequently induce the VEGF release from cocultured cells.

Recent research suggests that exosomes play an important role in cell-cell communications, and proteomic analyses of nasal mucosa-derived exosomes identified multiple proteins and microRNAs involved in immune responses, the coagulation system, and tissue remodeling(25-27). NLF-derived exosomes were shown to induce the migration of monocytes, neutrophils, and NK cells(28), and NLF-derived exosomes from patients with CRSwNP were shown to promote angiogenesis and vascular permeability in human umbilical vein endothelial cells(29). NLF-derived and nasal epithelial cell-derived exosomes from patients with CRSwNP were shown to reduce the proliferation of human nasal epithelial cells(30). Exosomes derived from IL-13-treated bronchial epithelial cells were shown to induce the proliferation of monocytes to a greater degree than exosomes from control epithelial cells(31). Intraperitoneal injection of GW4869 significantly suppressed exosome production in BALFs in house dust mite-induced asthmatic mice, and inhibited eosinophil infiltration and the production of IL-4 and IL-13 in BALFs(32). However, few reports have focused on fibroblast-derived exosomes in the pathophysiology of airway tissue remodeling.

In the present study, NPF-derived EVs were isolated and visualized using TEM, which revealed that the morphology and size of NPF-derived EVs were similar to exosomes of previous reports(16). We then examined the effect of isolated EVs on the release of VEGF. NPF-derived EVs (exosomes concentration: 25 pg/mL) significantly stimulated the release of

VEGF from EoL-1 eosinophilic leukemia cells. Pretreatment of NPFs with GW4869 or DMA inhibited coculture-induced release of exosomes and VEGF. These results indicate that NPF-derived exosomes are important in the coculture-induced release of VEGF. Fibroblasts are a major source of VEGF in airway inflammation, and NPFs produce VEGF in response to a variety of stimuli, such as hypoxia, viral infection, lipopolysaccharides, tumor necrosis factor-α, and prostaglandins(33-37). However, NPF-derived EVs had no effect on the release of VEGF from NPFs. This is the first report demonstrating a role of NPF-derived exosomes in tissue remodeling associated with sinonasal inflammation. In the lower airway, bronchial fibroblast-derived exosomes from severe asthmatic patients reportedly stimulate the proliferation of bronchial epithelial cells to a greater degree than bronchial fibroblast-derived exosomes from control subjects(38).

In the present study, the mechanism by which eosinophil-NPF interactions induce the release of exosome from cocultured cells is unclear. The role of eosinophil-derived mediators on exosome secretion is unknown. Several studies have shown that exosome secretion was induced by cellular stresses such as irradiation, hypoxia and anticancer drug, cisplatin. Induction of endoplasmic reticulum (ER) stress caused by tunicamycin also enhanced exosome secretion (39). We confirmed that hypoxia stimulated exosome secretion from cultured NPFs using an airtight culture container and anaeropack (data not shown). It is not clear why cells respond to stress by releasing more exosomes. Secreted exosomes may affect neighboring cells and possibly induce pathological conditions(39). These results suggest that cellular stresses caused by eosinophil-fibroblast interactions may induce exosome secretion. Further research is necessary to study the mechanism of exosome secretion from the cells under various stresses.

Exosomes are known to be released from eosinophils and to regulate the functions of eosinophils, epithelial cells, and muscle cells in asthmatic patients(40, 41). Eosinophil-

derived exosomes from asthmatic patients were shown to induce the production of nitric oxide and reactive oxygen species in eosinophils and stimulate the migration and adhesion of eosinophils(40). Asthmatic eosinophil-derived exosomes were shown to induce the apoptosis of epithelial cells and proliferation of muscle cells(41). In order to exclude effects associated with bioactive substances, serum-free medium was used for the coculture system in the present study. Eosinophils and EoL-1 cells released almost no exosomes in this serum-free and growth factor-free medium. Eosinophils are known to be activated by the exosomes released by themselves via autocrine and paracrine mechanisms. These results suggest that eosinophil-derived exosomes are also involved in the release of VEGF from eosinophils via autocrine and paracrine mechanisms, although the amounts of exosomes in cell supernatants were much less than those of NPFs.

This study has three main limitations. Firstly, because of the limited number of collected blood eosinophils, EoL-1 cells were used in many parts of this experiment, and the number of cultured blood eosinophils (100,000/mL) is ten times lower than EoL-1 cells (1,000,000/mL). We confirmed that coculture-induced VEGF release from peripheral blood eosinophils and NPFs began to increase at 3 h of incubation (data not shown), but time-course study using blood eosinophils was incapable. NPF-derived EVs stimulate VEGF release from EoL-1 cells, but did not stimulate that from normal blood eosinophils. Secondly, the proportion of exosomes released from NPFs and eosinophils/EoL-1 cells is unclear, although it has been reported that exosomes are released from both cell types. Finally, the function of normal peripheral blood eosinophils may be different from activated tissue eosinophils or peripheral blood eosinophils from patients with CRSwNP. This possibility will be the subject of further studies.

In conclusion, the present study demonstrated for the first time that eosinophil-fibroblast interactions induce the release of the profibrotic cytokine VEGF, which plays an important

role in the pathophysiology of tissue remodeling in conditions associated with eosinophil-predominant chronic airway inflammation, such as nasal polyposis and bronchial asthma. NPF-derived exosomes may play a crucial role in eosinophil-fibroblast interactions; thus, exosome inhibition may be a potential therapeutic target for the treatment of intractable airway inflammation.

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DISCLOSURE STATEMENT

The authors declare no competing financial interests.

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413

FIGURE LEGENDS

Figure 1. Coculture-induced release of exosomes and VEGF. Coculture of peripheral blood eosinophils and NPFs for 24 h stimulated the release of exosomes (A) and VEGF (B) (n=4). Coculture of EoL-1 cells and NPFs for 24 h stimulated the release of exosomes (C) and VEGF (D) (n=6). *P<0.05 when compared between coculture group and single cell group, one-way ANOVA with Tukey's honestly significant difference test or Games-Howell test.

Figure 2. Release of exosomes (A) and VEGF (B) over time in coculture of EoL-1 cells and NPFs for 3, 6, 12, and 24 h. Coculture-induced release of exosomes and VEGF was stimulated at 3 h of incubation. Coculture-induced exosome release continued to decrease for up to 24 h of incubation, although coculture-induced VEGF release continued to increase for up to 24 h of incubation (n=6). *P<0.05 when compared between coculture group and EoL-1 cell group, one-way ANOVA with Tukey's honestly significant difference test or Games-Howell test.

Figure 3. Effect of isolated NPF-derived EVs on the release of VEGF from EoL-1 cells. (A) Size and concentration of NPF-derived EVs as determined by nanoparticle tracking analysis. (B) Transmission electron microscopy of NPF-derived EVs. (C) Isolated NPF-derived EVs (exosome concentration: 25 pg/mL) significantly stimulated VEGF release from EoL-1 cells (n=4). *P<0.05, unpaired t-test.

Figure 4. Effects of GW4869 or DMA on coculture-induced release of exosomes and VEGF. Pretreatment of NPFs for 18 h with GW4869 (10 μM), an inhibitor of exosome production, significantly attenuated the release of exosomes (A) and VEGF (B) from

439 cocultured EoL-1 cells and NPFs at 24 h (n=6). Pretreatment of NPFs for 18 h with DMA
440 (10 μg/mL), an inhibitor of exosome secretion, significantly attenuated the release of
441 exosomes (C) at 6 and 24 h and VEGF (D) at 3, 6, and 24 h from cocultured EoL-1 cells
442 and NPFs at 3, 6, and 24 h (n=4). *P<0.05, unpaired t-test.

Fig. 1

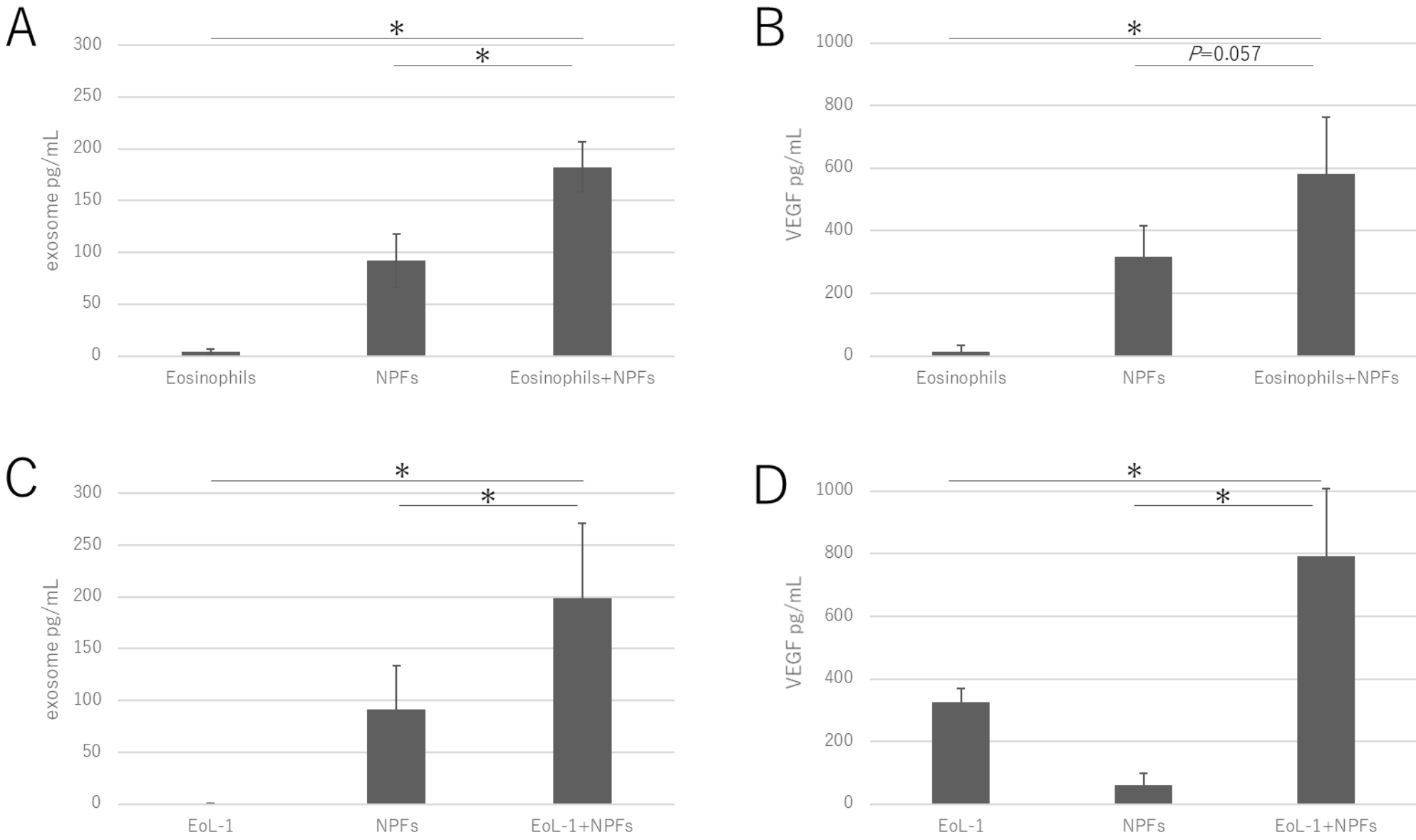

A
exosome pg/mL
300
250
200
150
100
50
0
*
*
Eosinophils
NPFs
Eosinophils+NPFs
B
VEGF pg/mL
1000
800
600
400
200
0
*
P=0.057
Eosinophils
NPFs
Eosinophils+NPFs
C
exosome pg/mL
300
250
200
150
100
50
0
*
*
EoL-1
NPFs
EoL-1+NPFs
D
VEGF pg/mL
1000
800
600
400
200
0
*
*
EoL-1
NPFs
EoL-1+NPFs

Fig. 2

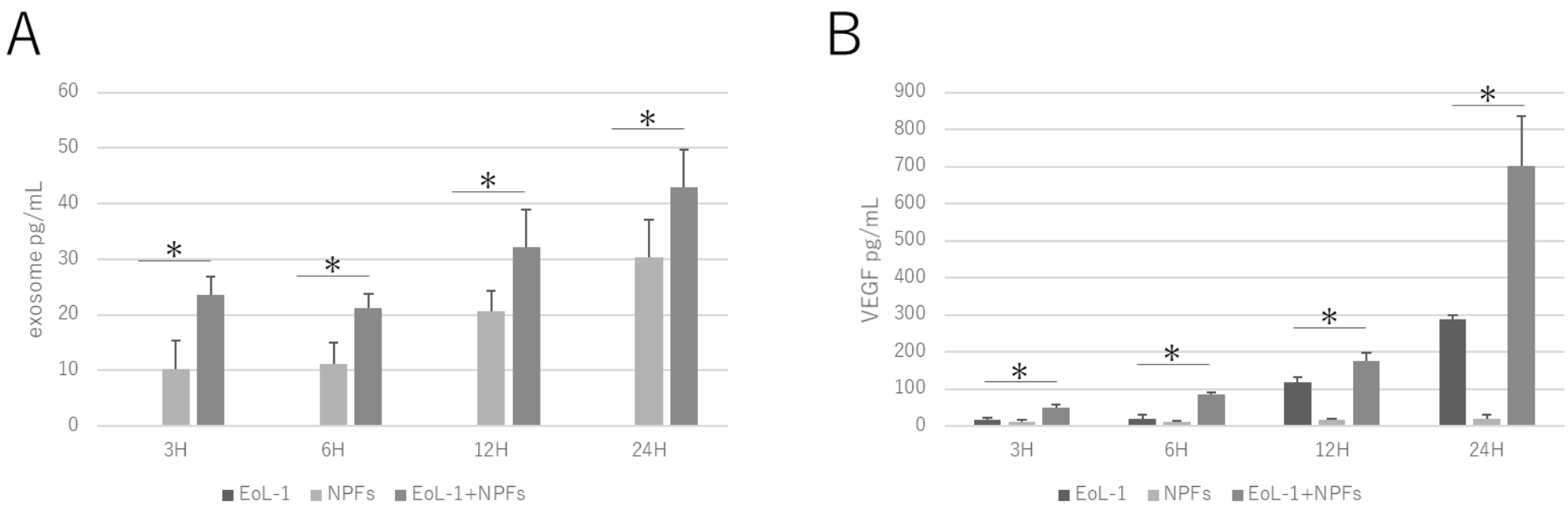
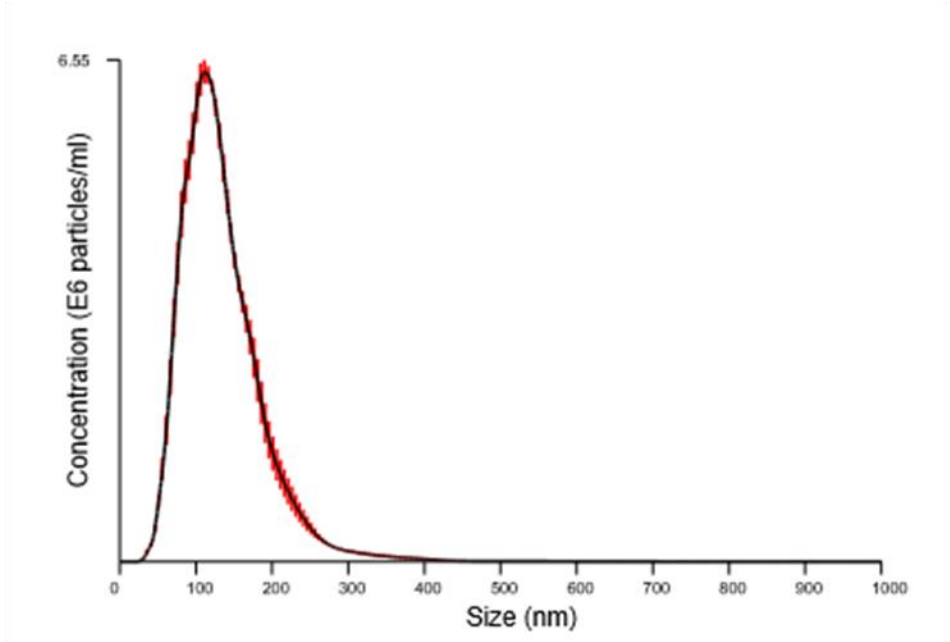
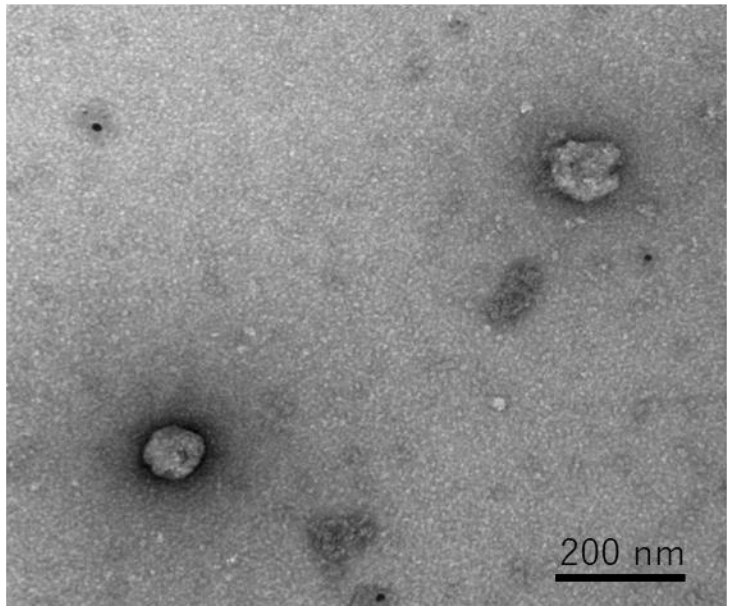

A
60
50
40
30
20
10
0
exosome pg/mL
*
*
*
*
3H
6H
12H
24H
EoL-1
NPFs
EoL-1+NPFs
B
900
800
700
600
500
400
300
200
100
0
VEGF pg/mL
*
*
*
*
3H
6H
12H
24H
EoL-1
NPFs
EoL-1+NPFs

Fig. 3

A


6.55
Concentration (E6 particles/ml)
0
100
200
300
400
500
600
700
800
900
1000
Size (nm)

B


200 nm

C

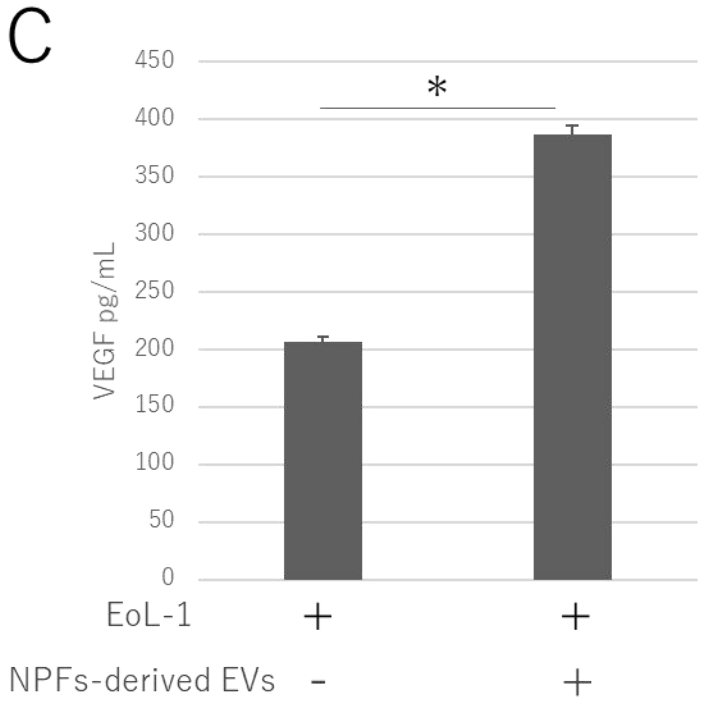
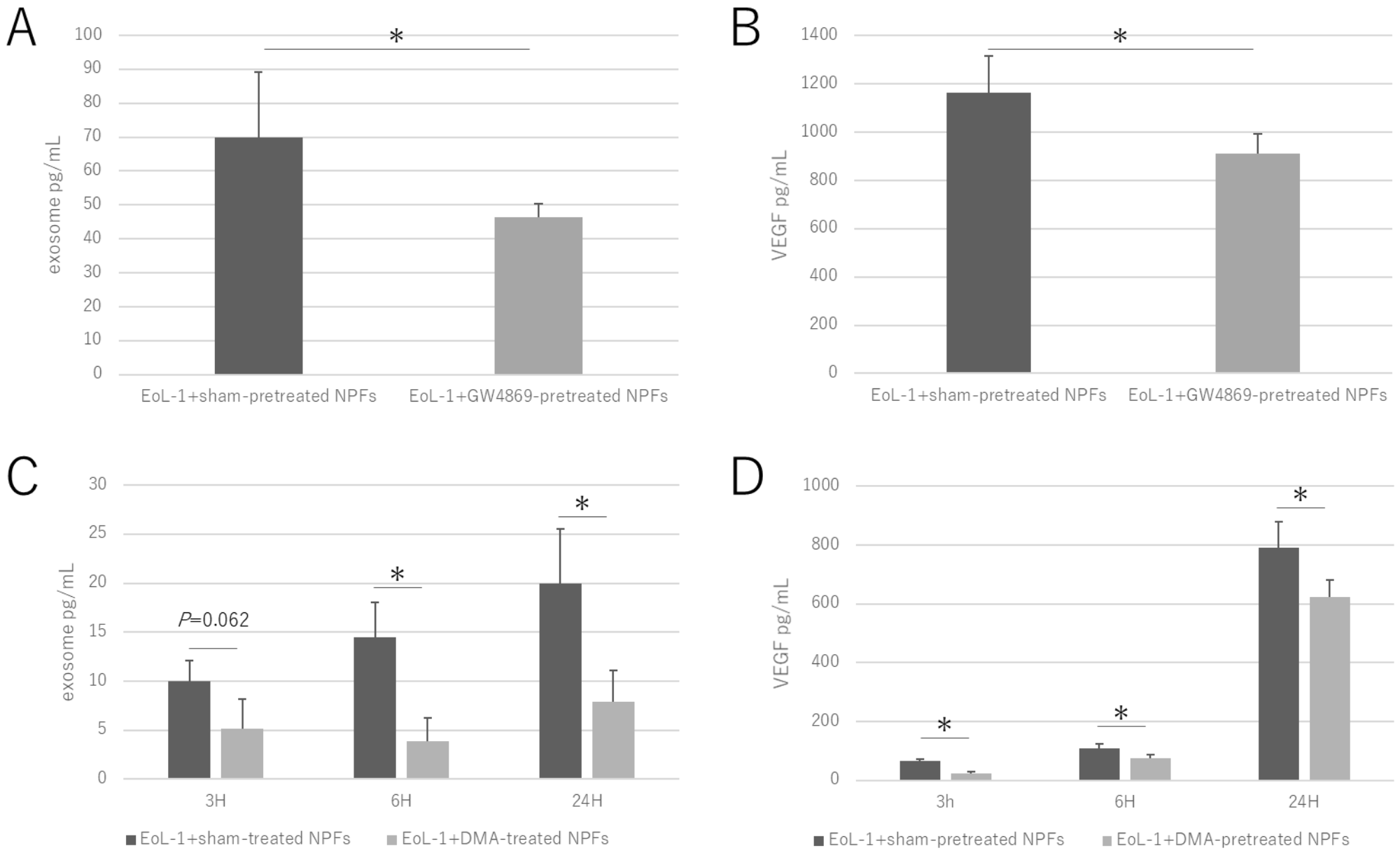

450
400
350
300
250
200
150
100
50
0
VEGF pg/mL
*
EoL-1
+
+
NPFs-derived EVs
-
+

Fig. 4


A
exosome pg/mL
100
90
80
70
60
50
40
30
20
10
0
*
EoL-1+sham-pretreated NPFs
EoL-1+GW4869-pretreated NPFs
B
VEGF pg/mL
1400
1200
1000
800
600
400
200
0
*
EoL-1+sham-pretreated NPFs
EoL-1+GW4869-pretreated NPFs
C
exosome pg/mL
30
25
20
15
10
5
0
P=0.062
*
*
3H
6H
24H
EoL-1+sham-treated NPFs
EoL-1+DMA-treated NPFs
D
VEGF pg/mL
1000
800
600
400
200
0
*
*
*
3h
6H
24H
EoL-1+sham-pretreated NPFs
EoL-1+DMA-pretreated NPFs