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Original Article

Nucleolin positively regulates spontaneous cell proliferation but is not involved in inhibition of proliferation by lead in cultured bovine aortic endothelial cells

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ABSTRACT — Nucleolin (NCL) is an abundant DNA-, RNA-, and protein-binding protein that is expressed ubiquitously in eukaryotic cells, implicating it in many cellular functions such as gene silencing, senescence, cytokinesis, and proliferation. NCL is also involved in angiogenesis events including vascular endothelial cell migration and proliferation. We previously found that lead, an environmental pollutant with vascular toxicity, inhibits the repair process of injured vascular endothelium *via* suppression of cell proliferation as a result of a lower proliferative response of cells to endogenous fibroblast growth factor-2. The present study investigated the expression of NCL in proliferating bovine aortic vascular endothelial cells and the role of NCL in proliferation of the cells in the presence or absence of lead. We found that the expression of NCL protein was independent of cell density. Both siRNA-mediated NCL knockdown and an anti-NCL-neutralizing antibody inhibited the proliferation of vascular endothelial cells without non-specific cell damage, indicating that NCL was involved in endothelial cell proliferation. However, lead significantly inhibited the proliferation of vascular endothelial cells without changing the expression level of NCL. Therefore, NCL may positively regulate spontaneous proliferation, but is not involved in the inhibition of proliferation by lead in vascular endothelial cells.

Key words: Lead, Nucleolin, Vascular endothelial cell, Atherosclerosis, Proliferation

INTRODUCTION

Nucleolin (NCL) is a multifunctional protein that is distributed ubiquitously in various eukaryotic cell compartments, especially the nucleolus (Tajrishi *et al.*, 2011). NCL functions as a prominent RNA-binding protein to interact with precursor ribosomal RNA (rRNA) and is essential for rRNA biogenesis and its transport to the cytoplasm (Ginisty *et al.*, 1999; Bouvet *et al.*, 2001). It has also been implicated in many cellular functions such as gene silencing, senescence, cytokinesis, nucleogenesis, cell proliferation, and growth (Jia *et al.*, 2017). Previous studies have demonstrated involvement of cell surface NCL in tumor growth and angiogenesis (Destouches *et al.*, 2008; Birmpas *et al.*, 2012). Additionally, cell surface NCL is a specific marker of angiogenic endothelial

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cells (Christian *et al.*, 2003). Numerous proteins involved in angiogenesis have been shown to interact with cell surface NCL, such as vascular endothelial growth factor (VEGF) (Jia *et al.*, 2017). The expression of NCL is also enhanced on the surface of endothelial cells upon stimulation by VEGF, and functional blockade or downregulation of the expression of cell surface NCL in vascular endothelial cells significantly inhibits the migration of endothelial cells and prevents the formation of capillary tubules (Huang *et al.*, 2006). Therefore, NCL may play an important role in vascular endothelial cell proliferation and migration.

Lead is a harmful heavy metal that causes a range of health problems including dysfunctions of nervous, renal, and vascular systems (Patrick, 2006). In particular, lead exposure has been shown to be an important determinant of hypertension in human population studies (Navas-Acien *et al.*, 2007). Several animal studies have also suggested that, in addition to cadmium and arsenic, lead is a risk factor of atherosclerosis (Vaziri, 2008; Prozialeck *et al.*, 2008). Vascular endothelial cells play an important role in regulation of vascular functions and lead-induced endothelial dysfunctions result in atherosclerosis, thrombosis, and tissue injury (Shinkai and Kaji, 2012). We have previously shown that lead exerts adverse effects on vascular endothelial cells, such as inhibition of the repair process of wounded monolayers *via* suppression of cell proliferation (Kaji *et al.*, 1995; Fujiwara *et al.*, 1997). We also found that inhibition of the repair of injured vascular endothelium by lead is due to a lower proliferative response of the cells to endogenous fibroblast growth factor-2 (Fujiwara and Kaji, 1999a), which is caused by suppression of heparan sulfate proteoglycan perlecan synthesis (Fujiwara and Kaji, 1999b). However, the molecular mechanisms of inhibition of endothelial cell proliferation by lead have not been fully elucidated.

Several reports have shown an interrelationship between alteration of NCL expression and arteriosclerosis (Li *et al.*, 2017; Kinumi *et al.*, 2005). Oxidative stress and oxidized low-density lipoprotein (LDL), which cause dysfunction in vascular endothelial cells, also induce NCL downregulation in cultured human umbilical vein endothelial cells (Zhang *et al.*, 2010; Kinumi *et al.*, 2005). These data raise the possibility that NCL influences the inhibitory effect of lead on vascular endothelial cell proliferation. Therefore, the present study investigated the change in expression of NCL in proliferating bovine aortic vascular endothelial cells and the role of NCL in cell proliferation and the lead-induced growth inhibitory effect.

MATERIALS AND METHODS

Materials

Vascular endothelial cells derived from bovine aorta were purchased from Cell Applications (San Diego, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Nissui Pharmaceutical (Tokyo, Japan) and Biowest (Nuaillé, France), respectively. TrueLine cell culture dishes and plates were purchased from Nippon Genetics (Tokyo, Japan). CytoTox96® non-radioactive cytotoxicity assay, a lactate dehydrogenase (LDH) assay kit, was purchased from Promega (Madison, WI, USA). ISOGEN II reagent was obtained from Nippon Gene (Tokyo, Japan). ReverTra Ace qPCR RT Master Mix and THUNDERBIRD SYBR qPCR Mix were purchased from Toyobo (Osaka, Japan). PCR primers for *NCL* (sense, 5′-AAAGGTTGCAGTTGCCACAC-3′ and anti-sense, 5′-TGCTTTGGCAGGTGTAACTG-3′) and β-actin (*ACTB*) (sense, 5′-AAAGAGATCACTGCCCTGGC-3′ and anti-sense, 5′-ACTCCTGCTTGCTGATCCAC-3′) were purchased from Star Oligo Rikaken (Aichi, Japan). Double-stranded control small interfering RNA (siRNA) and an *NCL* siRNA (target sequence: GGAAGUUUGGCUAUGUGGAdTdT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies against NCL and β-actin were purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and Abcam (Cambridge, UK), respectively. Anti-mouse IgG-horseradish peroxidase was purchased from GE Healthcare (Tokyo, Japan). Lead nitrate [$Pb(NO_3)_2$], L-lactate dehydrogenase, and other reagents were obtained from Fujifilm Wako Pure Chemical (Osaka, Japan).

Cell culture and treatment

Cell culture was performed as described previously (Wu *et al.*, 2020). Briefly, bovine aortic vascular endothelial cells were seeded in six-well culture plates at 2×10^4 cells/cm^2 and then cultured for 24, 48, and 72 hr in DMEM supplemented with 10% FBS (10% FBS-DMEM). Phase contrast imaging was performed using a DMi1 inverted microscope (Leica Microsystems, Wetzlar, Germany). In some experiments, vascular endothelial cells were transfected with control siRNA or *NCL* siRNA, as described below, and then incubated for 24 hr in fresh serum-free DMEM. In other experiments, vascular endothelial cells were seeded at 1×10^4 cells/cm^2 and then cultured for 24 hr in 10% FBS-DMEM. After incubation, the cells were treated with an ani-NCL antibody (10 μg/mL) or lead (1, 2, 5, or 10 μM) for 24 hr in fresh serum-free DMEM. Additionally, confluent cul-

tures of vascular endothelial cells were treated with lead (2 or 5 μM) for 24 hr.

siRNA transfection

Vascular endothelial cells were transfected with siRNAs using Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA), in accordance with the manufacturer's protocol, as we performed previously (Takahashi *et al.*, 2020). Briefly, double-stranded siRNA (final siRNA concentration: 5 pmol) was added to RNAiMAX transfection reagent, followed by incubation for 10 min at room temperature to allow siRNA/RNAiMAX complex formation. After the incubation, the siRNA/RNAiMAX complex was added to a suspension of vascular endothelial cells (2.5×10^5 cells/mL). The transfected cells were seeded at 400 μL per well in 24-well culture plates (5×10^4 cells/cm^2) and then incubated for 48 hr.

Real-time RT-PCR analysis

Total RNA extraction from cultured cells and subsequent real-time RT-PCR analysis were performed as described previously (Takahashi *et al.*, 2018). Briefly, after incubation, the culture medium was removed, the cell layer was washed twice with cold Ca- and Mg-free phosphate-buffered saline (CMF-PBS), and cold ISOGEN II reagent was added to each culture well. Cells were collected by scraping and homogenized by pipetting. The RNA quality was ensured by spectrophotometric analysis (OD260/280) using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed using ReverTra Ace qPCR RT Master Mix and the GeneAmp PCR system 9700 (Thermo Fisher Scientific). Real-time PCR was performed with THUNDERBIRD SYBR qPCR Mix using 0.5 μM primers and a LightCycler 96 (Roche, Tokyo, Japan). Fold changes in the *NCL* mRNA level were determined after normalization of the intensity value to that of *ACTB*. Data are represented as relative mRNA levels to the control set as 1.

Western blot analysis

Western blot analysis was performed as described previously with minor modifications (Takahashi *et al.*, 2020). Briefly, after incubation, the cell layers were washed with cold CMF-PBS and lysed with RIPA buffer. After collection, the solution was centrifuged, the supernatant was collected, and the protein concentration was determined. Protein samples were separated by SDS-polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane (Merck KGaA, Darmstadt, Germany), and visualized using primary antibodies against NCL and β-actin, and anti-mouse IgG-horseradish peroxidase as the secondary antibody.

Determination of cell numbers

The number of cells was counted as described previously (Kaji *et al.*, 1995). Vascular endothelial cells were seeded in 24-well culture plates at 1×10^4 cells/cm^2 and then cultured for 24 hr in 10% FBS-DMEM. After culture, the cells were treated with lead (1, 2, 5, or 10 μM) for 24 hr in fresh serum-free DMEM and then harvested with CMF-PBS containing 0.1% trypsin and 0.02% EDTA. The harvested cell suspension was thoroughly pipetted and the number of viable cells was counted with a hemocytometer after addition of the same volume of a 4% trypan blue solution.

LDH leakage assay

An LDH leakage assay was used to estimate cytotoxicity as described previously (Fujiwara *et al.*, 2016). After incubation with lead, siRNAs, or the anti-NCL antibody, the conditioned medium was harvested, and an aliquot of the medium was used to measure LDH activity with the CytoTox96® non-radioactive cytotoxicity assay kit. Absorbance of each sample was measured at 490 nm using a Multiskan FC microplate reader (Thermo Fisher Scientific). LDH activity (IU/L) of each sample was calculated based on a calibration curve using a standard LDH solution.

Statistical analysis

All statistics were performed using Excel software (Microsoft, Redmond, WA, USA) with the Statcel3 add-in (OMS, Tokyo, Japan). Data are expressed as the mean ± S.D. Statistical significance of the data was determined using one-way ANOVA with the Bonferroni/Dunn *post-hoc* test or Student's *t*-test as appropriate. *P*-values of less than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Figure 1 shows the time course of the NCL protein level in proliferating vascular endothelial cells. The cells were seeded at 2×10^4 cells/cm^2 and then incubated for up to 72 hr in 10% FBS-DMEM. As shown in Fig. 1A, sparse, subconfluent, and confluent cell layers were formed at 24, 48, and 72 hr, respectively, after cell seeding. As shown in Fig. 1B, the intensity of the NCL band was constant under these conditions, suggesting that the NCL protein level was maintained independently of the density of vascular endothelial cells.

Next, we examined the effects of siRNA-mediated

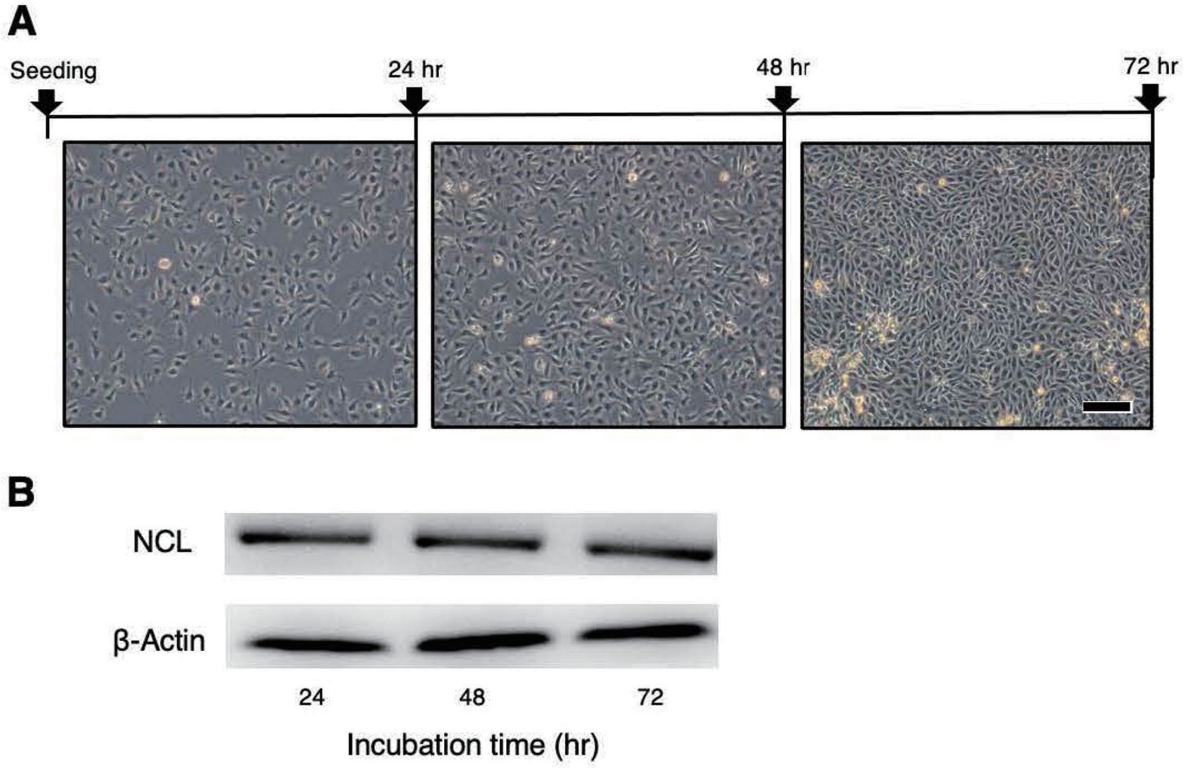

Fig. 1. Time course of the NCL protein level in proliferating vascular endothelial cells. Bovine aortic vascular endothelial cells were seeded at 2×10^4 cells/cm^2 and then incubated for 24, 48, and 72 hr. [A] Scheme of the experiment and a representative image of vascular endothelial cells at each time point. Scale bar = 200 μm. [B] NCL protein levels were determined by western blot analysis.

NCL knockdown on the proliferation of vascular endothelial cells. The knockdown efficiency of NCL in the cells is shown in Fig. 2A. The expression levels of *NCL* mRNA were reduced significantly by approximately 80% in *NCL* siRNA-transfected cells after 48 hr compared with control siRNA-transfected cells. After incubation for 24 hr, the number of viable cells was decreased in significantly sparse cultures of *NCL* siRNA-transfected cells compared with control siRNA-transfected cells (Fig. 2B). Similarly, the viable number of sparse vascular endothelial cells was decreased significantly by the anti-NCL antibody (Fig. 2C). At this time, there were no cytotoxic effects of NCL knockdown and the anti-NCL antibody in vascular endothelial cells (Fig. 2D and E). These results indicated that NCL was involved in the spontaneous proliferation of vascular endothelial cells.

We have previously shown that lead inhibits the proliferation of vascular endothelial cells without non-specific cell damage (Kaji *et al.*, 1995). Similar results were confirmed in sparse cells treated with lead for 24 hr in the present study as shown in Fig. 3A and B. The *NCL* mRNA level was not changed after 6 hr, but increased significantly after treatment with 5 and 10 μM lead for 24 hr in a concentration-dependent manner (Fig. 3C). However, lead did not increase the expression of NCL protein in the cells (Fig. 3D). These results suggested that lead inhibited vascular endothelial cell proliferation without elevation of the intracellular NCL level. Although responses of vascular endothelial cells are often dependent on cell density (Kaji *et al.*, 2000; Hara *et al.*, 2020), the level of both *NCL* mRNA and protein was not changed by lead in dense cultures of vascular endothelial cells (Fig. S1).

Previous reports have been shown that oxidative stress and oxidized LDL induce NCL downregulation in human umbilical vein endothelial cells (Zhang *et al.*, 2010; Kinumi *et al.*, 2005). Adenosine diphosphate treatment for 48 hr or more downregulates the level of NCL protein and inhibits the proliferation of human umbilical vein and aortic endothelial cells, (Wang *et al.*, 2014). Although it is possible that long-term exposure to lead may reduce NCL levels in vascular endothelial cells, the present data suggested that NCL was not involved in lead-induced inhibi-

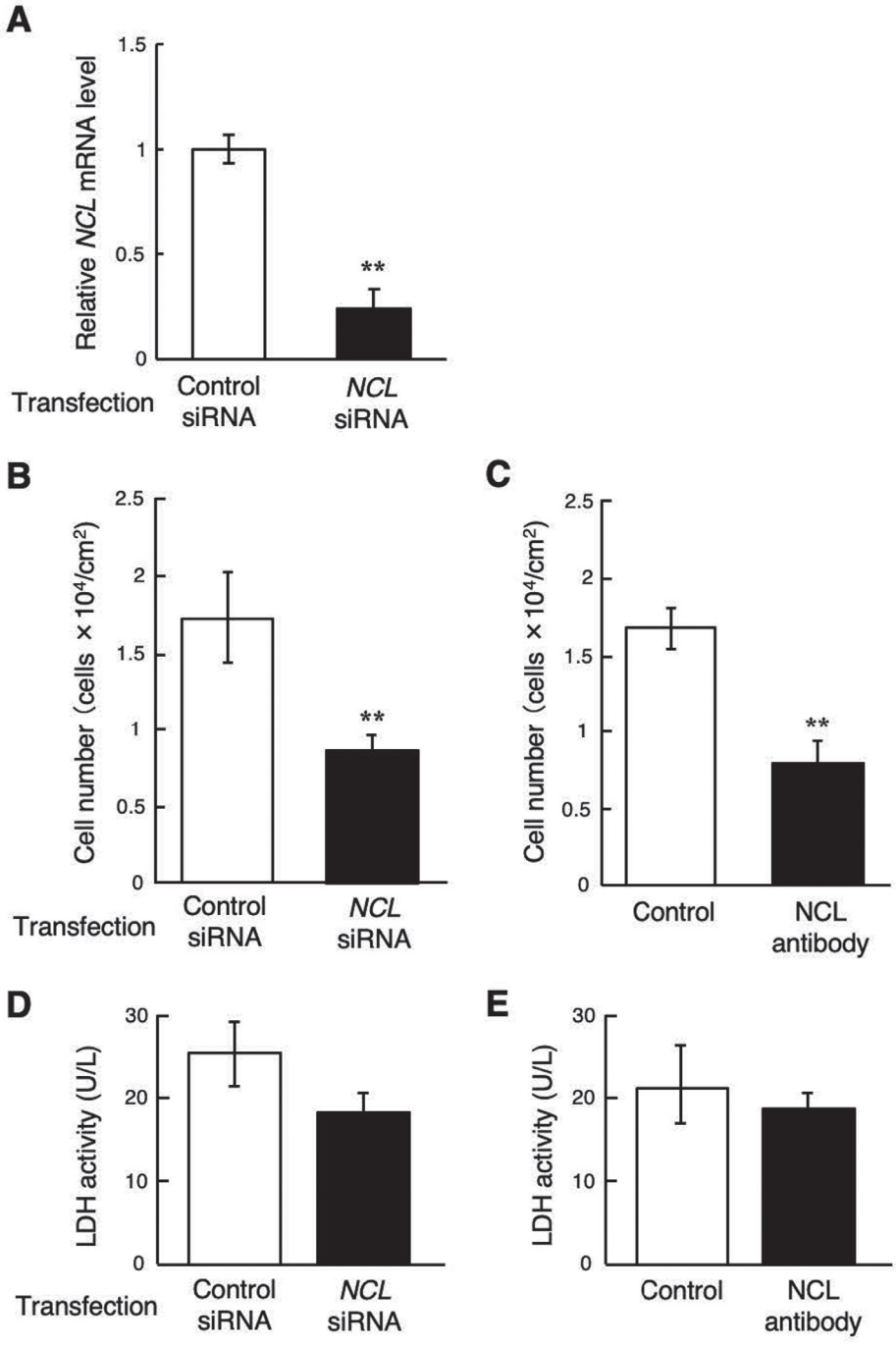

Fig. 2. Effects of NCL knockdown and the anti-NCL antibody on the proliferation of vascular endothelial cells. [A] Knockdown efficiency of *NCL* mRNA in bovine aortic vascular endothelial cells transfected with control or *NCL* siRNAs for 48 hr. Values are means ± S.D. (n = 3). Significantly different from the control siRNA group, $^{**}p < 0.01$. [B] Vascular endothelial cells transfected with control or *NCL* siRNAs for 48 hr were incubated in serum-free medium for 24 hr. The cell number was determined using a hemocytometer. Values are means ± S.D. (n = 3). Significantly different from the control siRNA group, $^{**}p < 0.01$. [C] Number of vascular endothelial cells treated with the anti-NCL antibody for 24 hr. Values are means ± S.D. (n = 4). Significantly different from control, $^{**}p < 0.01$. [D] Cytotoxic effects of NCL knockdown were determined by the LDH leakage assay. Data are represented as the mean ± S.D. (n = 3). [E] Cytotoxic effects of the anti-NCL antibody in vascular endothelial cells. Data are represented as the mean ± S.D. (n = 4).

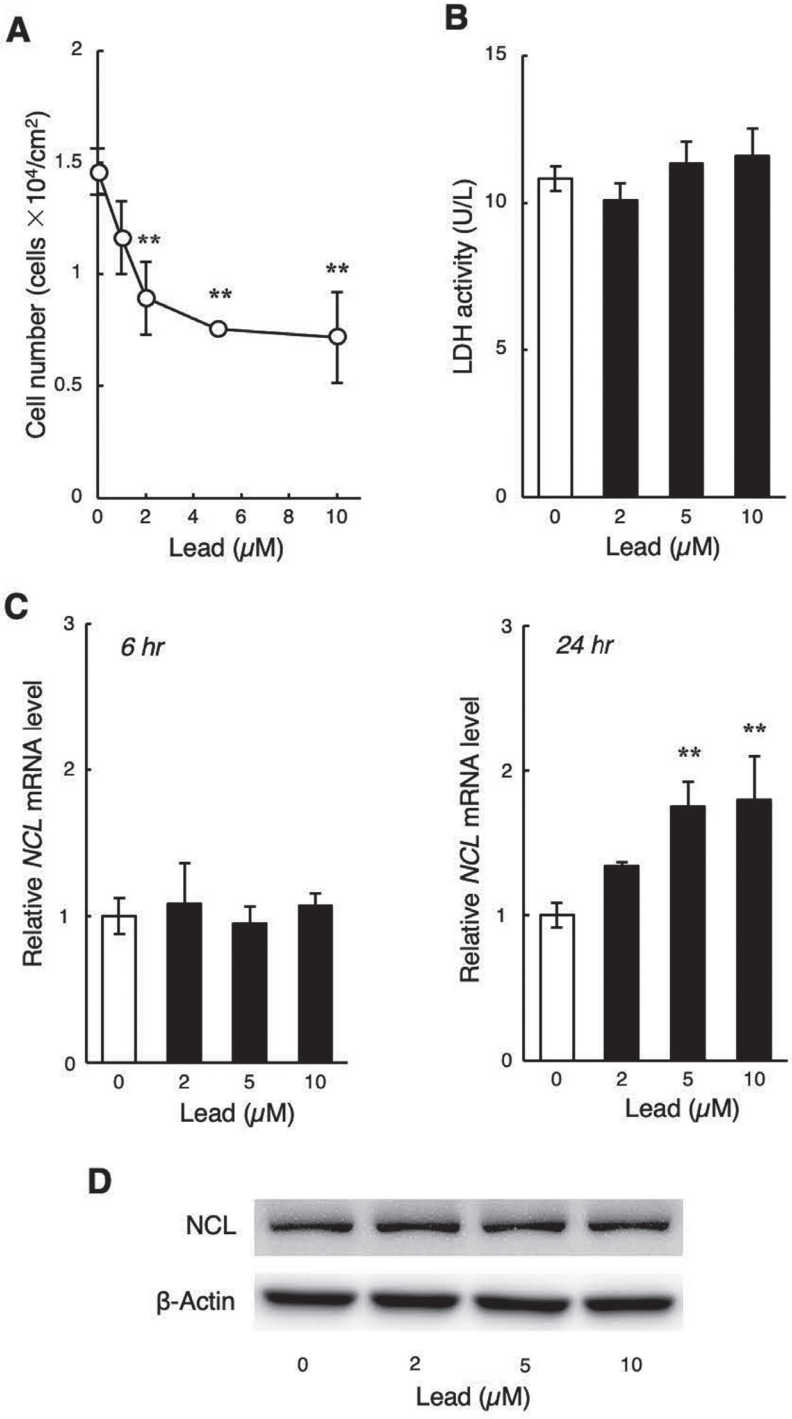

Fig. 3. Effects of lead on proliferation, cytotoxicity, and NCL expression of vascular endothelial cells. Bovine aortic vascular endothelial cells were treated with lead (1, 2, 5, or 10 μM) for 6 (C) or 24 hr (A, B, C, and D). [A] Effect of lead on the proliferation of vascular endothelial cells. The number of cells after treatment with lead for 24 hr is shown. Values are means ± S.D. (n = 4). Significantly different from the control, $^{**}p < 0.01$. [B] Cytotoxic effects of lead in vascular endothelial cells. Data are represented as the mean ± S.D. (n = 4). [C] *NCL* mRNA levels in cells treated with lead for 6 hr (left) or 24 hr (right). Values are means ± S.D. (n = 3). Significantly different from the control, $^{**}p < 0.01$. [D] NCL protein levels were determined by western blot analysis.

tion of vascular endothelial cell proliferation. Therefore, NCL positively regulates spontaneous cell proliferation, but is not involved in the inhibition of proliferation by lead in vascular endothelial cells.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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