Table 4 *(continued)*

Item	*Category*	*Exfoliated epithelial membrane*		*Total*	*p Value*
		No	*Yes*		
Bile acid cholagogue	Yes	2	0	2	1.000
	No	52	16	68	
Soporific antidepressant agent	Yes	4	0	4	0.567
	No	50	16	66	
Antihyperlipidaemic agent	Yes	4	0	4	0.567
	No	50	16	66	
Bone calcium metabolic agent	Yes	6	0	6	0.325
	No	48	16	64	
Analgesic	Yes	4	0	4	0.567
	No	50	16	66	
Antiallergic agent	Yes	2	0	2	1.000
	No	52	16	68	

of the tongue dorsum) and open mouth as independent variables and formation of peeling epithelial membrane as the dependent variable. As a result, the adjusted odds ratio of dryness of the tongue dorsum was 32.3 (95% confidence interval: 36.8–271.9, $p = 0.001$), and the odds ratio of open mouth was 25.8 (95% confidence interval: 2.2–298.7, $p = 0.009$, Table 5).

Discussion

The membranous substances that formed in the mucous membranes of the palate were derived from the oral mucosa. This membranous substance has been identified as a crust[2,3], sputum[4–7] or a peeling epithelial membrane. Crusts are materials that that cover the surfaces of wounds, are exposed to the air and are dried exudates and/or concretions containing a high proportion of red blood cells. Red blood cells were not found to be abundant in the sampled membranous substances. The main constituent was, instead, the degenerated keratin product derived from stratified squamous epithelium that consisted of layered keratinised substances that were cytokeratin-1-positive. This means that the

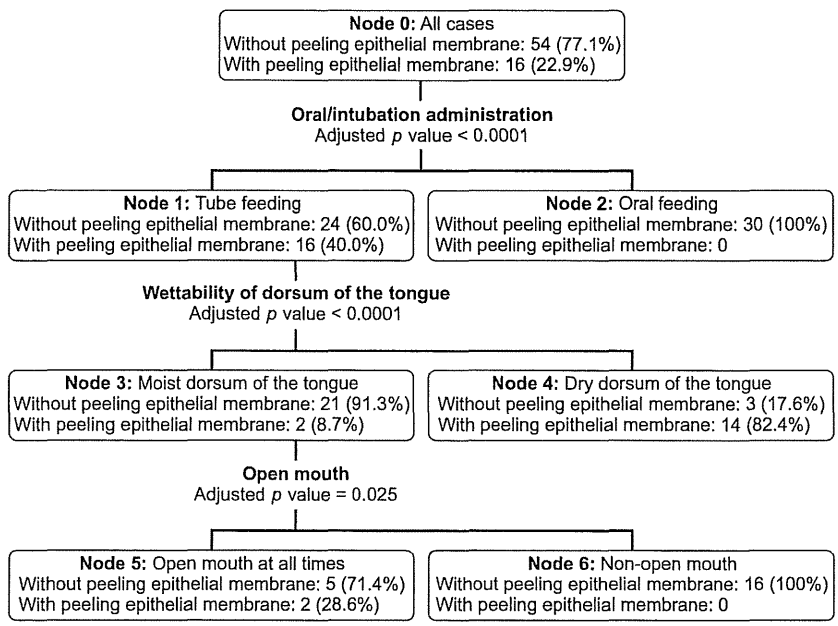

Figure 4 Formative factors for peeling epithelial membrane according to a tree analysis (tree diagram).

Table 5 The outcome of a logistic regression analysis

Explanatory variable	*Adjusted odds ratio*	*95% Confidence interval*	*p Value*
Dryness of dorsum of the tongue	32.3	3.8–271.9	0.001
Open mouth	25.8	2.2–298.7	0.009

peeling epithelial membranes sampled in this study were different from crusts.

Sputum is an airway secretion containing mucus, peeled epithelium from the respiratory tract, inflammatory cells, etc., and is expectorated by coughing. Epithelium contained in sputum is derived from the epithelium of the pharynx, the trachea and the bronchi and is histologically characterised as multiple rows of ciliated columnar epithelium. No ciliated columnar epithelium was observed in the sampled membranous substances. There are some cases in which squamous epithelial metaplasia is observed due to respiratory diseases and expectoration. However, it is normally columnar epithelium and/or small amounts of stratified squamous epithelium metamorphosis that accompanies the sputum in non-specific inflammatory responses, and layered structures with a predominance of keratin are never observed[17]. Moreover, although the epiglottis, aryepiglottic fold and vocal cords consist of stratified squamous epithelia, the sampled membranous substances were such that they covered a wide area of the palate and were believed to be different from the stratified squamous epithelium of the larynx, which is usually localised to the larynx.

The membranous substances observed in this study shift and adhere to the normal epithelium of the mucous membrane of the palate, potentially causing bleeding when removal is attempted using a pair of tweezers. Membranous substances were observed in the mouth of patients with impaired consciousness, but patients with impaired consciousness had no cough reflex, and it was difficult to determine whether the membranous substances had been expectorated inside the mouth due to coughing and had adhered to the palate. Instead, the membranous substances sampled in this study were believed to be peeling epithelial membranes that had shifted to stratified squamous epithelium inside the mouth. One substance found in the membranes other than the epithelial components was a homogeneous mucosubstance, probably proteins derived from mucin based on a previous report[8]. This was believed to be derived from saliva secreted from the salivary gland inside the mouth and nasal mucus secreted from the goblet cells of the nasal cavity and/or nasal gland. Although the main constituents are epithelial components, the presence of membranous substances consisting of both peeling epithelial membranes and mucosubstances is a risk factor for bleeding, mucous membrane inflammation and suffocation due to the exfoliation falling into the pharynx. Dental health professionals caring for elderly patients must, therefore, attempt to prevent the formation of these membranous substances.

Although the levels of correlation between variables may be determined by multivariate logistic regression, this method cannot reveal the relationships between these factors. In this study, multivariate logistic regression was used to investigate the level of correlation, but a decision tree analysis was used to investigate the relationship between the factors related to the formation of membrane-like substances. As a result, the number one factor found to be correlated with formation of the membranes was the method of eating. No membranous substances were found in any of the patients with oral ingestion. The membranous substances were only observed in those patients who were fed through a nasal tube or gastric fistula, and this is believed to be a requirement for the formation of membranous substances, not just a risk factor. In patients who eat orally, saliva spreads around the mouth due to chewing, speech, tongue movement, strains on the buccal mucosa, etc., and the oral mucosa, including mucous membranes, becomes wet. This prevents the epithelium of the palate from drying out and prevents the formation of membranous substances. Even in patients who are fed by tubes, the formation of membrane-like substances may be suppressed by commencing ingestion after training to eat and swallow foods.

The risk factor with the second highest level of correlation with membrane formation was dryness of the tongue dorsum. Compared to patients with a wet tongue dorsum, those with a dry tongue dorsum had a 32.3-fold greater tendency towards formation of the membranous substance. Of the16 patients in whom membranous substances were sampled, 14 patients had non-oral ingestion and experienced dryness of the tongue dorsum. This suggests that non-oral ingestion was a requirement for the formation of the membranous substances and that dryness of the tongue dorsum was the subsequent major cause of

formation of the membranous substances. There was no correlation with the wettability of the mucous membrane below the tongue; therefore, non-oral ingestion and dryness of the tongue dorsum imply a dryness of the oral mucosa that is independent from the amount of saliva. It has been reported that there is a high level of correlation in elderly patients between having a dry tongue dorsum and being bedridden or tube-fed[18]. Our results show that formation of the membranous substances was also correlated with speech and communication. Thus, loss of oral cavity function in elderly patients requiring nursing care results in drying of the tongue dorsum, and this becomes a risk factor for the formation of membranous substances in the palate. We believe that the membranous substances are formed when there is no movement of the tongue, cheeks or jaws, because of which saliva does not wet the oral mucosa. This leads to dryness of the tongue dorsum and drying out of the mucous membrane of the palate.

Wettability of the palate is related to the secretional capacity of the major salivary gland[19]. Although salivation still occurs from the sublingual caruncle and opening of the parotid gland, if the tongue, jaw and cheeks do not move, the saliva accumulates under the tongue and does not lead to wetting of the tongue dorsum. Thus, the mechanism for the formation of membranous substances in the mucous membrane of the palate is believed to be dehydration of the mucosal epithelium due to dried oral mucosa, dehydration leading to weakening of epithelial cell–cell adhesion in the stratum corneum and drying of the adhered salivary component accompanied by peeling of the epithelium and formation of a film. There is a possibility that inflammation subsequently occurs in the peeled area. Peeling epithelial membranes are believed to form via the same mechanism as senile xerosis, in which the stratum corneum dries and becomes rough, and adhesion between corneum layers is weakened, causing peeling[20,21].

The third risk factor for the formation of membranous substances was a continuously open mouth. Even when the dorsal side of the tongue was damp upon tubal feeding, membrane-like substances tended to form in patients in whom the mouth was open at all times. We believe that in patients who have an open mouth at all times, the mucous membrane of the palate is exposed to the air, and drying of the mucous membrane of the palate occurs, leading to formation of the peeling epithelial membrane.

Of the 70 items investigated in this study, no disorders or commonly used medicines were found to be risk factors associated with the formation of membranous substances. This suggested that these membranes were not indicative of any particular disorder, and they were not caused by any commonly used medications.

The results of this study suggest that using moisturising agents to prevent the oral mucosa from drying might suppress formation of the membrane-like substances in patients with limited or no oral activity. It is therefore believed to be important to educate workers in elderly wards and institutes, as well as families with elderly people requiring nursing care, regarding tubal feeding, assisted tooth brushing and the application of moisturisers to prevent the formation of membrane-like substances. Furthermore, the formation of membrane-like substances may be prevented by having tube-fed patients ingest their food orally, so it is also important to conduct training for eating and swallowing after evaluating the eating and swallowing capabilities of the tube-fed patients.

Preventing the formation of membrane-like substances remains a significant issue that needs to be addressed in the case of patients requiring oral care. In clinical situations, there is a danger of swallowing portions of the epithelium that peel off during oral care and a danger of bleeding caused by injury inside the mouth due to removal of the peeling epithelial membranes. Treatment by a dentist is necessary for removing peeling epithelial membranes that have formed, and guidance on preventing formation is also necessary.

Conclusion

The method of food ingestion was highly correlated with the formation of membranous substances in the palates of elderly persons requiring nursing care. Our results suggest that non-oral ingestion was the primary factor for such membrane formation. Dryness of the tongue dorsum and a continuously open mouth were highly correlated with membrane formation in patients who were tube-fed. Altogether, our results suggest that impairment in oral cavity function in terms of chewing and speaking is the root cause of formation of the membranous substances and that moisturising the oral cavity can prevent their formation.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number 24593177 (Principal Investigator:

Tadashi Ogasawara), the Integrated Research Project Number 10103578 (Principal Investigator: Yasuaki Kakinoki) and 22150501 (Principal Investigator: Yasuaki Kakinoki).

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RESEARCH REPORTS
Biological

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J Dent Res 92(3):241-246, 2013

A. actinomycetemcomitans LPS Enhances Foam Cell Formation Induced by LDL

ABSTRACT

The objective of this study was to examine whether native low-density lipoprotein (LDL) induces foam cell formation by macrophages and to examine the effect of lipopolysaccharide (LPS) on native LDL-induced foam cell formation by macrophages *in vitro*. RAW 264.7 cells were cultured with LDL or high-density lipoprotein (HDL) in the presence of LPS derived from *Aggregatibacter actinomycetemcomitans*. Foam cell formation was determined by staining with Oil-red-O to visualize cytoplasmic lipid droplet accumulation. The expression of LDL-receptor and the degree of internalization of FITC-conjugated LDL in RAW 264.7 cells were examined by immunofluorescence microscopy. The images were digitally recorded and analyzed with Image J software. Statistical analysis was performed by JMP software. Foam cell formation was induced by the addition of native LDL in dose- and time-dependent manners, whereas HDL showed no effect. LPS enhanced the foam cell formation induced by native LDL. In addition, LPS stimulated the expression of LDL-receptor protein on RAW 264.7 cells and enhanced the internalization of LDL. The enhancement of foam cell formation induced by LPS and LDL was inhibited by the depolymerizing agent nocodazole and amiloride analog 5-(N-ethyl-N-isoprophyl) amiloride (EIPA). Our findings indicate that LPS plays an important role in foam cell formation by LDL-stimulated macrophages.

KEY WORDS: periodontopathic bacteria, macrophage, LDL-receptor, macropinocytosis, atherosclerosis, periodontitis.

DOI: 10.1177/0022034512473309

Received August 17, 2012; Last revision November 6, 2012; Accepted December 10, 2012

INTRODUCTION

Atherosclerosis shows a chronic inflammatory process initiated by the recruitment of monocyte-derived macrophages into the subendothelial arterial space and their transformation into lipid-laden foam cells (Ross, 1993). Low-density lipoprotein (LDL) is an abundant atherogenic lipoprotein in plasma (Pyörälä *et al.*, 1994) and the main component of plasma cholesterol, which enters vessel walls and then, by some mechanism, enters macrophages. Previously, macrophage foam cell formation was reported to occur only through the uptake of modified forms of LDL such as oxidized or aggregated LDL (Kruth, 2001). However, a previous study demonstrated that macrophage foam cell formation can occur *via* the uptake of native LDL (Kruth *et al.*, 2002). Chronic bacterial infections, including periodontitis, are associated with an increased risk of coronary heart diseases (Pussinen *et al.*, 2003; Spahr *et al.*, 2006; Sakurai *et al.*, 2007). However, the mechanisms by which chronic infections increase the likelihood of atherosclerosis are poorly defined.

Periodontitis is a chronic inflammatory disease characterized by the destruction of tooth-supporting structures. *Aggregatibacter actinomycetemcomitans*, a Gram-negative periodontopathic bacterium, plays a crucial role in the development of periodontal disease (Nishihara and Koseki, 2004). Lipopolysaccharide (LPS) in periodontal pockets promotes atherogenesis as an inflammatory agent by activating leukocytes. The atherogenic properties of LPS derived from *A. actinomycetemcomitans* and their effects on macrophage-like cells, namely, RAW 264.7 cells, have been examined in several studies (Pussinen *et al.*, 2004; Lakio *et al.*, 2006). In addition, previous experimental and clinical investigations have indicated that adhesion of monocytes to vascular endothelial cells is an important step in atherosclerosis (Libby *et al.*, 2002). Macrophages play an important role in the pathogenesis of atherosclerosis and are one of the major cell types involved in foam cell formation. In contrast, scant attention has been paid to the effects of periodontopathic bacterial LPS on foam cell formation induced by LDL. In the present study, we examined the synergistic effect of LPS on LDL-stimulated macrophages.

MATERIALS & METHODS

Cell Culture

The murine macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained at 37°C in α-minimal essential medium (α-MEM; GIBCO BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum

(FBS), penicillin G (100 U/mL), and streptomycin (100 μg/mL) in an atmosphere of 5% CO_2 in air.

LPS Preparation

LPS was extracted from lyophilized cells of *A. actinomycetemcomitans* Y4 by a hot phenol-water method (Nishihara *et al.*, 1986). The extract was treated with nuclease and washed extensively with pyrogen-free water by ultracentrifugation. The major chemical components of the extract were 41% neutral sugar, 8% hexosamine, and 31% fatty acid (Tsutsumi *et al.*, 2010).

Cell Viability Assays

Cell viability was determined with the tetrazolium salt WST-1 (4-[3-(4- iodophenyl)-2H-5-tetrazolio]-1-3-benzene disulfonate) (Wako Pure Chemical Industries, Ltd., Osaka, Japan). RAW 264.7 cells were plated in 96-well culture plates at a concentration of 2 x 10^4/mL and treated with LDL (Sigma-Aldrich Co., St. Louis, MO, USA) or high-density lipoprotein (HDL) (Sigma-Aldrich) solution for 44 hrs. These lipoproteins were derived from human plasma, dissolved in 150 mM NaCl, pH 7.4, supplemented with 0.01% EDTA and stored at 4°C. Endotoxin in these lipoproteins was below the detection level as determined by Limulus amebocyte lysate assay (data not shown). WST-1 solution (10 μL) was added to each well, followed by incubation for 4 hrs. Absorbance at 450 nm and 630 nm was measured by means of a Multiskan JX microplate reader (Thermo ELECTRON CO., Yokohama, Japan).

Macrophage Foam Cell Formation and Lipid Staining

We examined the foam cell formation by macrophages using staining with Oil-red-O to visualize cytoplasmic lipid droplet accumulation. RAW 264.7 cells were plated in 96-well culture plates at a concentration of 2 x 10^4/mL at 1 day before stimulation, then stimulated with LDL or HDL in the presence or absence of nocodazole (Calbiochem, San Diego, CA, USA) or 5-(N-ethyl-N-isoprophyl) amiloride (EIPA, Sigma-Aldrich) for various time periods. In some experiments, the cells were precultured with LPS for 24 hrs prior to stimulation with LDL, nocodazole, and EIPA. The cells were fixed with 10% formaldehyde for 10 min, washed with phosphate-buffered saline (PBS; pH 7.2), and dehydrated with 60% isopropanol for 1 min, followed by staining with a working solution of Oil-red-O (Primary Cell Co., Ltd., Sapporo, Japan) for 20 min. After cells were washed in 60% isopropanol in PBS, foam cell formation by macrophages was observed under an optical microscope (IX71; Olympus Co., Tokyo, Japan), and images were captured digitally in real time by means of a CCD camera.

Immunofluorescent Staining

The expression of LDL-receptor (LDL-R) in RAW 264.7 cells was examined by immunofluorescence microscopy. RAW 264.7 cells were plated in 8-well chamber plates (Nalge Nunc International, Rochester, NY, USA) at a concentration of 5 x 10^4/mL, then stimulated with LDL or HDL (100 μg/mL) for various time periods. Next, the cells were rinsed with PBS and then fixed with 4% formaldehyde for 15 min at room temperature. After being washed in PBS, the cells were blocked with blocking buffer (PBS supplemented with 1% bovine serum albumin), followed by incubation with anti-LDL-R polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4°C. Bound antibodies were detected with Alexa Fluor 488 anti-goat IgG antibody (Invitrogen, Carlsbad, CA, USA) for 2 hrs at room temperature. The cells were visualized by means of a Fluorescence Microscope BZ-9000 (KEYENCE Corp., Osaka, Japan). Images were captured digitally in real time and processed with BZ-II imaging software (KEYENCE Corp.; magnification, x 40; exposure time, 2.0 sec). The images were digitally recorded and stored as 'tagged image file format' (TIFF) files. Semi-quantitative analysis of the detected proteins was performed by histogram analysis with Image J software (http://rsbweb.nih.gov/ij, National Institutes of Health, Bethesda, MD, USA). Briefly, each image was first color-split into a single green channel image, followed by thresholding at a value of 0 to 255. The volume of the LDL-R-positive area was then measured as the sum of the pixel number multiplied by the brightness for each level. The average volume of control samples was assigned the value 1.0. At least 30 cells in 5 to 10 fields *per* dish were recorded, and values obtained from 3 dishes on different days were averaged for each time-point.

LDL Labeling

LDL (1 mg/mL) in 0.1 M $NaHCO_3$ (pH 9.0) was gently mixed with 10 μL/mL of FITC (2 mg/mL in dimethyl sulfide), then unbound dye was removed by gel filtration on a Sephadex G-25 column equilibrated with 150 mM NaCl (pH 7.4) and 0.01% EDTA buffer (Brinkley, 1992). RAW 264.7 cells (2.5 x 10^3/mL) were incubated with LPS for 24 hrs and added to FITC-conjugated LDL. After being cultured for an additional 12 hrs, the cells were visualized by means of a Fluorescence Microscope BZ-9000 (KEYENCE Corp.). Images were captured digitally in real time and processed with BZ-II imaging software (KEYENCE Corp.). Semi-quantitative analysis of the internalization was performed by histogram analysis with Image J software. The stained area was measured for 30 cells in 10 fields *per* dish and averaged for each time-point. The percentage of positive cells was calculated as internalized cell number/total cell number × 100.

Statistical Analysis

Statistical analysis of foam cell formation by macrophages was conducted with JMP software, version 8.0.2 (SAS Institute Inc., Cary, NC, USA). Results are expressed as the mean ± standard deviation (SD). One-way analysis of variance was used to analyze the manner in which the distribution of each continuous variable differed among the groups. A Tukey-Kramer HSD (honestly significant difference) test was utilized to examine differences in regard to group means. In immunofluorescence staining analysis, statistical significance was determined by the Dunnett test.

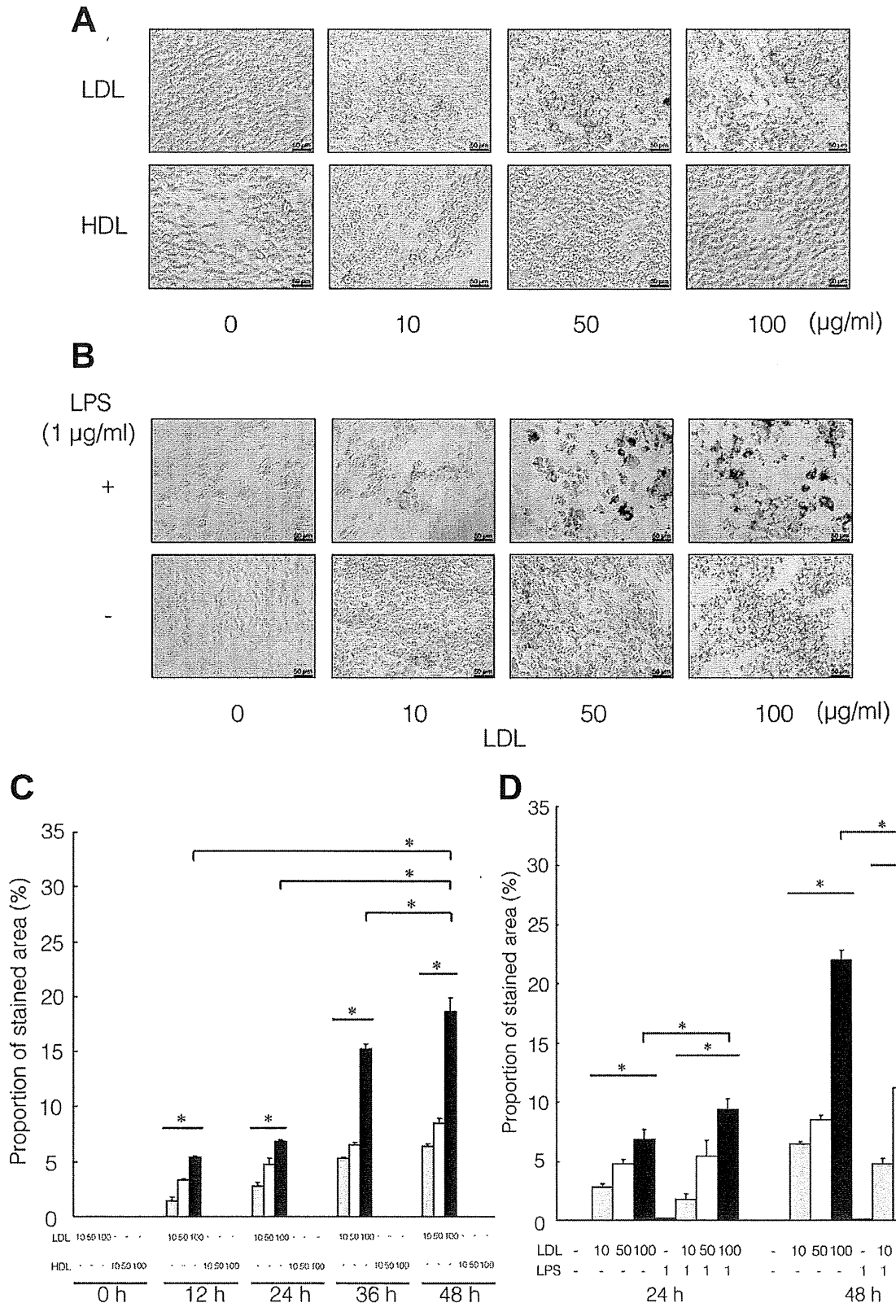

Figure 1. Representative microscope images of foam cell formation by macrophages. **(A)** RAW 264.7 cells were cultured with LDL or HDL at 37°C for 48 hrs, then fixed, stained with Oil-red-O, and visualized by optical microscopy. **(B)** RAW 264.7 cells were treated with LPS (1 μg/mL) for 24 hrs, then incubated with LDL for 48 hrs and analyzed by Oil-red-O staining for detection of foam cell formation. **(C, D)** The proportion (%) of stained areas in RAW 264.7 cells was calculated as described in Materials & Methods. Data are expressed as the mean ± SD of 3 experiments, with similar results obtained in each experiment. *Significant difference between mean stained areas of 2 groups, as assessed by the Tukey-Kramer HSD test ($p < 0.05$).

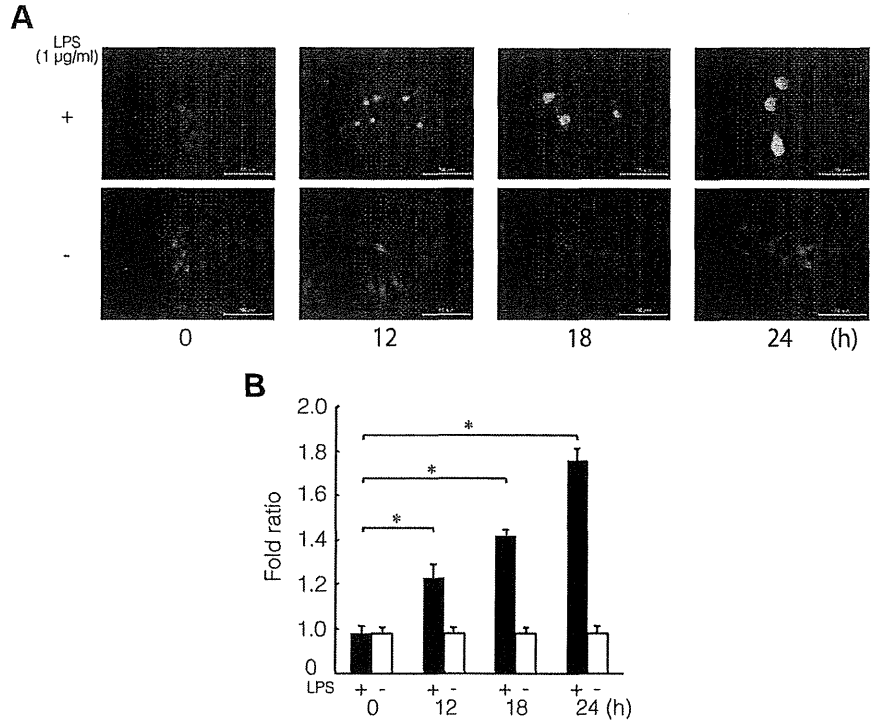

Figure 2. Expression of LDL-R in RAW 264.7 cells stimulated with LPS. **(A)** Cells were treated with LPS (1 μg/mL) at 37°C for the indicated times, then fixed and immunostained for LDL-R, and visualized by fluorescence microscopy as described in Materials & Methods. **(B)** Morphometric analysis of changes in LDL-R in RAW 264.7 cells. Data are expressed as the mean ± SD of 3 experiments, with similar results obtained in each. *Significant difference for mean of fold ratio between 2 groups, as assessed by the Dunnett test ($p < 0.05$).

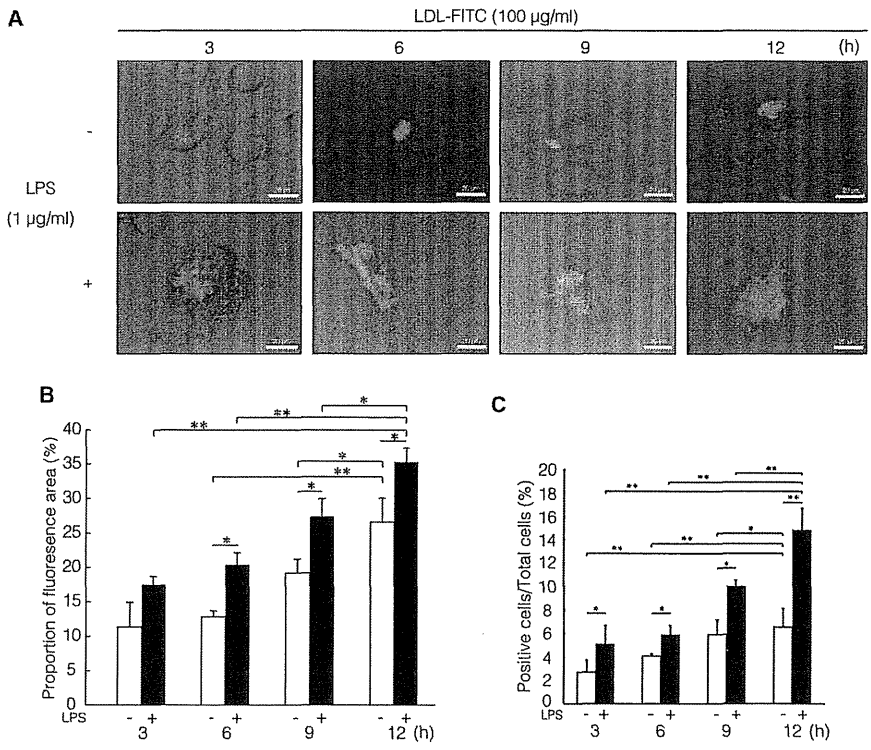

Figure 3. Internalization of LDL by macrophages. **(A)** RAW 264.7 cells were cultured with FITC-conjugated LDL in the presence of LPS and visualized by fluorescence microscopy as described in Materials & Methods. **(B)** The presence of LDL in RAW 264.7 cells was calculated as described in Materials & Methods. **(C)** The results were also expressed as a percentage (means ± SD) of the LDL-internalized cells. Data are expressed as the mean ± SD of 3 experiments, with similar results obtained in each.

RESULTS

Foam Cell Formation by Macrophages

We examined the effect of LDL on foam cell formation by macrophages. Treatment with LDL visually increased foam cell formation dose- and time-dependently, whereas HDL had almost no effect (Figs. 1A, 1C). To elucidate the effect of LPS on the foam cell formation induced by LDL, we treated RAW 264.7 cells with LPS (1 μg/mL) for 24 hrs, and then cultured them with LDL for 48 hrs. Treatment with LPS enhanced the foam cell formation by RAW 264.7 cells induced by LDL in a time-dependent manner up to 48 hrs, compared with the formation by RAW 264.7 cells stimulated with LDL in the absence of LPS (Figs. 1B, 1D).

Effect of LPS on LDL-R Expression

We examined the expression levels of LDL-R on RAW 264.7 cells stimulated with LPS. Immunofluorescence microscopic analysis revealed that the treatment with LPS (1 μg/mL) increased LDL-R expression in a time-dependent manner up to 24 hrs (Fig. 2).

Internalization of LDL into Macrophages

The degree of internalization of LDL into RAW 264.7 cells was examined by fluorescence microscopy. RAW 264.7 cells were pre-cultured with LPS (1 μg/mL) for 24 hrs, following the incubation with FITC-conjugated LDL (100 μg/mL) for an additional 12 hrs. FITC-conjugated LDL was internalized into macrophages when the cells were stimulated with LPS (Fig. 3).

Effects of Interfering Microtubes on Foam Cell Formation Induced by LDL

Enhancement of foam cell formation of RAW 264.7 cells induced by LDL and LPS was dramatically suppressed dose-dependently by the treatment with nocodazole (Fig. 4A) and EIPA (Fig. 4B). In contrast, nocodazole and EIPA had no effect on the cell viability of RAW 264.7 cells (data not shown).

DISCUSSION

Periodontitis is a bacterial infection with tooth-supporting tissue destruction, eventually leading to tooth loss. Gram-negative *A. actinomycetemcomitans* is involved in the pathology of several kinds of periodontitis. *A. actinomycetemcomitans* activates leukocytes because of expressing 2 exotoxins, a cytolethal distending toxin and a leukotoxin (Johansson, 2011). There is increasing evidence that foam cells present in atheromatous lesions are macrophages derived from circulating monocytes (Schaffner *et al.*, 1980; Gerrity, 1981). Several micro-organisms have been implicated in the infectious etiology of atherosclerosis (Haraszthy *et al.*, 2000), and several investigations have attempted to relate periodontitis to cardiovascular disease (Mattila, 2003; Cairo *et al.*, 2004). In the present study, we used a homogeneous clonal population of murine RAW 264.7 cells to elucidate the role of LPS derived from *A. actinomycetemcomitans* in the formation of foam cells by macrophages. This cell line is known to exhibit lipid accumulation and foam cell formation *via* scavenger receptor-mediated endocytosis and constitutive macropinocytosis (Yao *et al.*, 2009).

Although macrophage foam cell formation was reported to occur only through the uptake of modified forms of LDL, such as oxidized or aggregated LDL (Kruth, 2001), our results revealed that foam cell formation occurred through the uptake of native LDL (Fig. 1). A previous study reported that oxidized LDL induced apoptosis, reduced the viability of RAW 264.7 cells, and enhanced cell aggregation (Li *et al.*, 2006). Neither LDL nor HDL used in this study had an effect on the cell viability of RAW 264.7 cells. These results may exclude the possible involvement of the phenomenon that the oxidation of native LDL enhanced foam cell formation through cell aggregation. In addition, LPS was shown to induce further foam cell formation by macrophages in the presence of exogenous LDL, suggesting that periodontopathic bacteria are associated with increased risk of cardiac damage. *A. actinomycetemcomitans* is an important etiological agent of several kinds of periodontitis as well as endocarditis (Suzuki *et al.*, 2001). Chronic periodontitis is characterized by multiple episodes of bacteremia, which is an event that allows this pathogenic organism to migrate to areas of atherosclerotic plaque.

LDL-R functions as a primary receptor for binding and internalization of plasma-derived LDL cholesterol and regulates plasma LDL concentrations. The expressions of LDL receptors are subject to feedback control by intracellular cholesterol levels (Ye *et al.*, 2009). In the present study, we demonstrated that LPS significantly increased LDL-R protein expression (Fig. 2). Quantitative real-time reverse-transcription polymerase chain-reaction analysis also revealed that LPS stimulation increased in LDL-R mRNA copies as compared with untreated control RAW 264.7 cells (data not shown). This is consistent with a previous study which demonstrated that LPS significantly increased native LDL accumulation in THP-1 macrophages, specifically *via* the LDL-R pathway (Ye *et al.*, 2009). Taken together, these findings suggest that chronic inflammation may fundamentally modify cholesterol homeostasis by disrupting LDL receptor feedback regulation.

In addition to receptor-mediated endocytosis, macrophages possess high-level macropinocytosis activity (Steinman *et al.*, 1983). Foam cell formation by macrophages was previously thought to occur only by receptor-mediated uptake of LDL. However, recent studies have revealed that macropinocytosis is one of the important mechanisms for native LDL uptake and foam cell formation by macrophages (Kruth *et al.*, 2002, 2005; Anzinger *et al.*, 2010), as well as an actin-dependent process, including membrane ruffling and formation of membrane vesicles (Lee and Knecht, 2002). Nocodazole has been shown to partially inhibit macropinocytosis in mouse-derived macrophages (Racoosin and Swanson, 1992). It is well-known that nocodazole disrupts associations between microtubules and the plasma membrane, which normally permit macropinocytosis to occur. Moreover, the addition of EIPA, a specific inhibitor of macropinocytosis, Na^+/H^+ exchange blocker, also attenuated foam cell formation induced by LDL and LPS (Feng *et al.*,

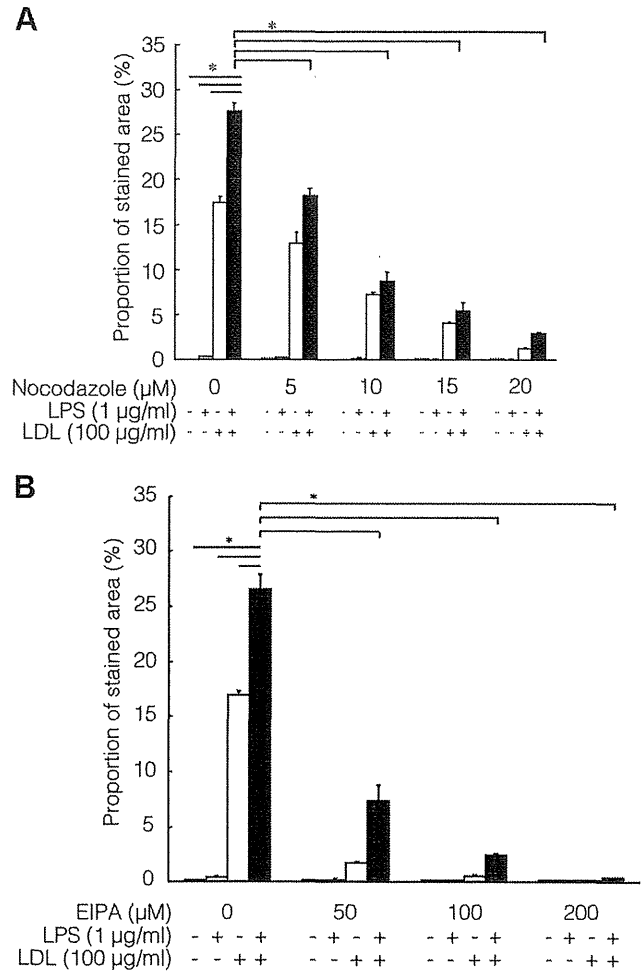

Figure 4. Inhibitory effects of nocodazole and EIPA on LDL-induced foam cell formation by macrophages. RAW 264.7 cells were pre-cultured with LPS (1 μg/ml) for 24 hrs, following the incubation with LDL (100 μg/ml) in the presence or absence of nocodazole and EIPA for 48 hrs. Foam cell formation was then analyzed by Oil-red-O staining. Staining rates are expressed as the mean ± SD of 3 experiments, with similar results obtained in each. *Significant difference between mean proportion of staining area in 2 groups, as assessed by the Tukey-Kramer HSD test ($p < 0.05$).

2010). We found that not only nocodazole, but also EIPA, remarkably inhibited the effect of LPS on LDL-induced foam cell formation by RAW 264.7 cells (Fig. 4). On the basis of these findings, we speculate that enhancement of LDL-induced foam cell formation by LPS is partially dependent on macropinocytosis.

In conclusion, our results show that LPS enhances the foam cell formation induced by LDL by activating LDL-R expression and macropinocytosis in RAW 264.7 cells. Although additional studies are needed to examine the correlation of native LDL and LPS with regard to etiology of atherosclerosis, by *in vivo* experiments, we propose that LPS derived from periodontopathic bacteria is involved in the induction of atherogenesis mediated by LDL.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science. The authors have received nothing of value from a commercial company or institution. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Biochemical and Biophysical Research Communications 420 (2012) 380–384

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mechanisms involved in inhibition of chondrogenesis by activin-A

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ARTICLE INFO

Article history:
Received 24 February 2012
Available online 8 March 2012

Keywords:
Activin-A
Chondrocyte
Smad
JNK
Akt

ABSTRACT

Objectives: Activin-A, a member of the TGF-β family, is known to be present in bone and cartilage. Although, involvement of the TGF-β family in chondrogenesis has been reported, the mechanism by which activin-A regulates chondrogenesis has not been fully elucidated. The aim of this study was to investigate the effects of activin-A on chondrocyte differentiation *in vitro*.

Materials and methods: Monolayer cultures of mouse chondrocyte ATDC cells were pretreated with a variety of inhibitors of major signaling pathways prior to addition of activin-A. The expressions of *sox9*, *runx2*, and *osterix* mRNA were detected using real-time PCR. To determine chondrocyte differentiation, sulfated glycosaminoglycans were stained with Alcian blue. To further elucidate the role of activin-A on chondrogenesis regulation, phosphorylation of Smad2/3, ERK, JNK, and Akt proteins was determined by western blotting.

Results: Activin-A suppressed the transcription of *sox9*, *runx2*, and *osterix* mRNA, as well as sulfated glycosaminoglycans accumulation. Activin-A also inhibited constitutive phosphorylation of JNK and Akt proteins. Furthermore, inhibition of the JNK and PI3K-Akt pathways by chemical inhibitors suppressed chondrogenesis in ATDC5 cells.

Conclusions: These results indicate that activin-A may suppress chondrocyte differentiation in ATDC5 cells via down-regulation of JNK and Akt phosphorylation.

1. Introduction

Osteoarthritis (OA), one of the most common joint disorders worldwide, is characterized by cartilage degeneration and osteophyte formation in joints. Articular cartilage has received much attention in OA studies, because gross articular cartilage damage is the most obvious pathologic feature leading to joint dysfunction. Recent studies have reported that endochondral ossification is an essential process in pathologic disorders such as osteophyte formation during OA progression [1,2].

In chondrogenesis, the transcription factor *sox9* is considered to be the master chondrogenic factor. *Sox9* is a member of a high-mobility-group DNA-binding domain that exhibits a high degree of homology with that of the mammalian testis-determining factor, *SRY* (*sex* determining region of Y chromosome) and is required for expression of such cartilage specific matrix proteins as collagen type II, IX, XI, and aggrecan [3,4]. Heterozygous mutations in the human *sox9* gene cause the skeletal malformation syndrome camptomelic dysplasia [5,6]. Studies in mice have shown that *sox9* is required for multiple steps along the chondrocyte differentiation pathway. In addition, *sox9*-deficient cells are known to not participate in mesenchymal condensation and do not express extra-cellular matrix (ECM) genes, which are known to be activated by *sox9* in chondrocytes [7]. *Sox9*-deficient cells are excluded from cartilage primordia throughout embryonic development [8]. In addition, cartilage is highly hypoplastic in *Col2a1-Cre-sox9*$^{flox/flox}$ conditional knockout mice, in which *sox9* expression is lost in condensed mesenchymal cells before differentiation into chondrocytes [9]. These findings suggest that expression of *sox9* in differentiated chondrocyte lineage cells is essential for cell survival.

Members of the transforming growth factor β (TGF-β) family are pleiotropic cytokines that are involved in inducing various kinds of cell differentiation [10]. Activins are closely related to their natural antagonists, inhibins, which are comprised of a common α subunit coupled to 1 of 2 β subunits leading to inhibin-A (αβA) or inhibin-B (αβB). Homo- and heterodimerization of the αβA and αβB subunits

Abbreviations: OA, osteoarthritis; SRY, sex determining region of Y chromosome; ECM, extracellular matrix; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; TGF-β, transforming growth factor β; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; FBS, fetal bovine serum; PI3K, phosphatidylinositol-3-kinase; PBS, phosphate-buffered saline; RT-PCR, reverse-transcription polymerase chain reaction; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; R-SMADs, regulatory transcription factors of the SMAD family; TAK1, TGF-β activated kinase-1.

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http://dx.doi.org/10.1016/j.bbrc.2012.03.003

result in formation of activin-A (βAβA), activin-AB (βAβB), or activin-B (βBβB) [11]. Although activin-A is known to be present in bone and cartilage, neither activin-B nor activin-AB have been detected in bone [12]. Although several studies have reported the role of activin-A in bone metabolism, conflicting evidence exists concerning the role of activin-A in bone formation and destruction, while it has also been reported to inhibit and stimulate osteoblastogenesis *in vitro*, and promote osteoclast formation *in vitro* [13–16]. *In vivo* studies have revealed that direct administration of activin-A increases bone mineral density [17], whereas overexpression of inhibin-A, which blocks activin-A and other TGF-β family members, increases bone mass [18,19].

Several researchers have reported involvement of the TGF-β family in chondrogenesis, though less attention has been paid to the impact of activin-A signaling on chondrocyte differentiation. In the present study, we examined the mechanisms by which activin-A inhibits chondrocyte differentiation in chondrogenesis.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 medium were obtained from Gibco BRL (Grand Island, NY, USA). Recombinant human activin-A was kindly provided by Institute for Innovation, Ajinomoto Co. (Kanagawa, Japan). Anti-phospho-Smad2 monoclonal, anti-Smad2/3 polyclonal, anti-phospho-extracellular signal-regulated kinase (ERK) monoclonal, anti-ERK polyclonal, anti-phospho-c-Jun N-terminal kinase (JNK) monoclonal, anti-phospho-Akt monoclonal, and anti-Akt polyclonal antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-JNK monoclonal antibody was obtained from R&D Systems Inc. (Minneapolis, MN, USA) and anti-β-actin monoclonal antibody was purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

Mouse chondrocyte ATDC5 cells were purchased from Riken Cell Bank (Ibaraki, Japan) and maintained in a 1:1 mixture of DMEM/Ham's F-12 medium containing 5% fetal bovine serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin, 10 μg/ml human transferring, and 3×10^{-8} M sodium selenite at 37 °C in an atmosphere of 5% CO_2.

ATDC5 cells were incubated in a serum-free 1% FBS for 24 h, then treated with FBS-free medium for the experiment, and incubated with activin-A for the indicated times. For signaling inhibition assays, ATDC5 cells were pre-treated with inhibitors of type I TGF-β receptor kinase (A-83-01, Santa Cruz Biotechnology, Santa Cruz, CA, USA), ERK (U0126, Calbiochem, San Diego, CA, USA); JNK (SP600125, Calbiochem), or phosphatidylinositol-3-kinase (PI3K)/Akt (LY294002, Calbiochem) for 1 h, then treated with activin-A for the indicated times in the presence or absence of each inhibitor.

2.3. Alcian blue staining

Chondrogenic differentiation of ATDC5 cells was determined by staining sulfated glycosaminoglycans with Alcian blue. ATDC5 cells (2.5×10^5 cells/ml) were seeded into 6-well plates, and cultured with activin-A (50 ng/ml) and human recombinant insulin (10 μg/ml) for 40 days. The medium was changed every 2 days. Cultured cells were washed and fixed with 4% paraformaldehyde at room temperature for 10 min. After washing in phosphate-buffered saline (PBS; pH7.2), the cells were incubated overnight at room temperature with Alcian Blue Solution (pH2.5; Nacalai tesque, Kyoto Japan). After being rinsed with distilled water, the results were recorded with a digital camera.

2.4. Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using an RNeasy Mini Kit (QIAGEN Inc., Valencia, CA, USA), according to the manufacturer's instructions. Obtained RNA was transcribed with SuperScript Reverse Transcriptase II (Invitrogen, Carlsbad, CA, USA) and amplified using a Mastercycler gradient (Ependorf AG, Hamburug, Germany). PCR products were detected using FAST SYBR® Green Master Mix (Applied Biosystems, Foster Cith, CA, USA) using the following primer sequences: *gapdh* forward: 5′-GACGGCCGCATCTTCTTGA-3′ and reverse: 5′-CACACCGACCTTCACCATTTT-3′, *runx2* forward: 5′-GCCGAGCTCCGAA ATGC-3′ and reverse: 5′-AGATCGTTGAACCTGGCTACTTG-3′, *osterix* forward: 5′-TCTGTTCCAAGCGCTTTACCA-3′ and reverse: 5′-CGTGGGTGCGCTGA TGT-3′, and *sox9* forward: 5′-ACCCACCACTCCCAAAACC-3′ and reverse: 5′-CGCCCCTCTCGCTTCAG-3′. Thermal cycling and fluorescence detection were performed using a StepOne™ Real-Time System (Applied Biosystems). The fold increase in copy numbers of mRNA was calculated as the relative ratio of the target gene to *gapdh*.

2.5. Western blotting analysis

The total protein was extracted using Cell Lysis Buffer (Cell Signaling Technology). Protein contents were measured using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Equivalent amounts of total protein per sample were electrophoresed using SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). Non-specific binding sites were blocked by immersing the membrane in 10% skim milk in PBS for 60 min at room temper-

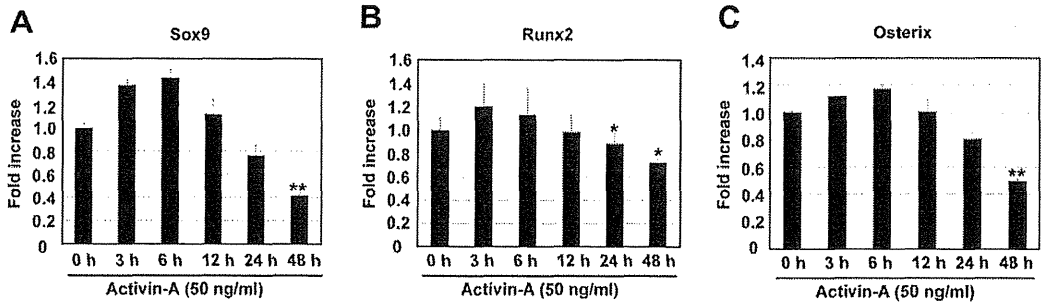

Fig. 1. Activin-A suppresses *sox9*, *runx2*, and *osterix* expression. ATDC5 cells were incubated with activin-A (50 ng/ml) for the indicated times, then total RNA was isolated and reverse-transcribed into cDNA using PCR amplification with SYBR green. PCR amplification was performed using primers specific for (A) *sox9*, (B) *runx2*, and (C) *osterix* and *gapdh*. Fold changes in *sox9*, *runx2*, and *osterix* mRNA copy number values shown represent the average ± SD of data derived from triplicate cultures. $^*P < 0.05$; $^{**}P < 0.01$, by Student's *t*-test.

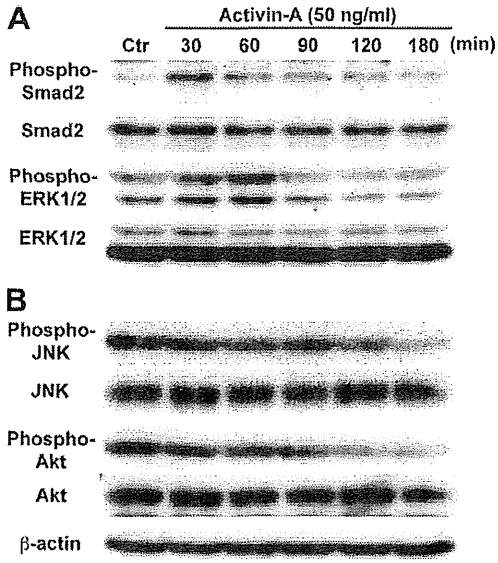

Fig. 2. Activin-A enhances smad2 and ERK phosphorylation, and suppresses JNK and Akt phosphorylation. ATDC5 cells were incubated with activin-A (50 ng/ml) for the indicated times, then whole cell lysates were analyzed by SDS–PAGE and western blotting analyses. (A) Expressions of phosphorylated Smad2 and ERK1/2. (B) Expressions of phosphorylated JNK and Akt. Following visualization, the western blots were stripped and re-probed for Smad2, ERK, JNK, Akt, and β-actin.

ature. After that, the membrane was washed 4 times with PBS, followed by incubation with the diluted primary antibody at 4 °C. Anti-phopsho-Smad2, anti-Smad2/3, anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-JNK, anti-JNK, anti-phospho-Akt, anti-Akt, and anti-β-actin were used as the primary antibodies, while horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG were used as secondary antibodies (GE Healthcare, Little Chalfont, UK). After washing the membranes, chemiluminescence was produced using ECL reagent (Amersham Pharmacia Biotech, Upspsala, Sweden).

2.6. Statistical analysis

Statistical differences were determined using an unpaired Student's *t*-test with Bonferroni's correction for multiple comparisons. *P* values less than 0.05 were considered significant. All data are expressed as the mean ± standard deviation (SD).

3. Results

3.1. Effect of activin-A on chondrocyte differentiation

To determine the effect of activin-A on chondrocyte differentiation, we assessed the mRNA expressions of *sox9*, *runx2*, and *osterix* as typical markers of chondrocyte differentiation. As early as after 24 h of incubation with activin-A, there was a decrease in *sox9* mRNA copies as compared to the untreated control chondrocytes (Fig. 1A). This effect of activin-A was time-dependent, with the

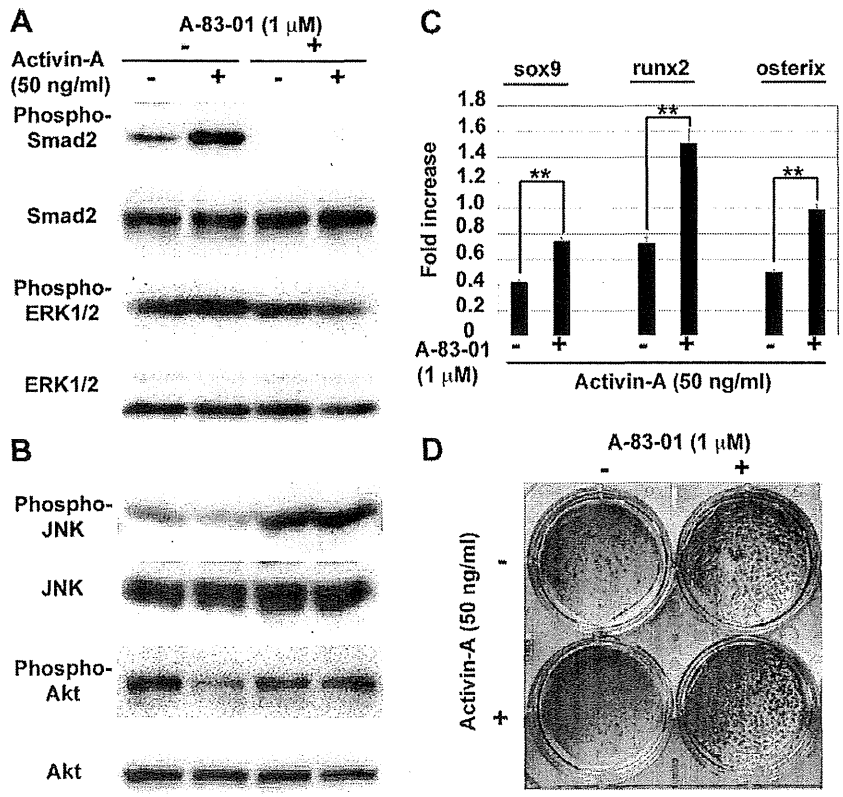

Fig. 3. Type I TGF-β receptor kinase inhibition recovered activin-A-dependent suppression of chondrogenesis. ATDC5 cells were pre-incubated with A-83-01 (1 μM) for 60 min as well as during the addition of activin-A (50 ng/ml) for the indicated times. Whole cell lysates were analyzed by SDS–PAGE and western blotting analyses. (A) Expressions of phosphorylated Smad2 and ERK1/2. (B) Expressions of phosphorylated JNK and Akt. Following visualization, the western blots were stripped and re-probed for Smad2, ERK, JNK, Akt, and β-actin. (C) Total RNA was isolated and reverse-transcribed into cDNA using PCR amplification with SYBR green. PCR amplification was performed using *sox9*, *runx2*, *osterix*, and *gapdh*. Fold changes in *sox9*, *runx2*, and *osterix* mRNA copy number values shown represent the average ± SD of data derived from triplicate cultures. $^{**}P < 0.01$, by Student's *t*-test. (D) ATDC5 cells were cultured with activin-A (50 ng/ml) in the presence or absence of A-83-01 (1 μM) for 40 days. Cultured cells were fixed with 4% paraformaldehyde and stained with Alcian blue.

maximum reduction observed after 48 h. As shown in Fig. 1B, C, treatment with activin-A also produced a statistically significant reduction in *runx2* and *osterix* mRNA expression by 24 h, and reached maximal reduction at 48 h.

3.2. Effect of activin-A on signal transduction

Next, we examined the phosphorylation levels of signaling molecules in the process of chondrogenic differentiation in ATDC5 by western blotting. Treatment with activin-A enhanced the amount of phosphorylated Smad2 (Phospho-Smad2) and ERK1/2 (phospho-ERK1/2) protein after 30 min (Fig. 2A). Furthermore, the levels of phosphorylated JNK (phospho-JNK) and Akt (phospho-Akt) were down-regulated following addition of activin-A in a time dependent manner up to 180 min (Fig. 2B). However, activin-A caused no change in p38 MAPK phosphorylation (data not shown).

3.3. Effect of type I TGF-β receptor kinase inhibitor on activin-A dependent suppression of chondrogenesis

To evaluate the role of Smad2, a key transducer of activin-A signaling in the regulation of chondrogenesis, ATDC5 cells were treated with type I TGF-β receptor kinase inhibitor (A-83-01) prior to stimulation with activin-A. The activin-A-induced phosphorylation of Smad2 and ERK was blocked by A-83-01 (Fig. 3A). In contrast, type I TGF-β receptor kinase inhibition prevented the inhibition of JNK and Akt phosphorylation by activin-A (Fig. 3B). Real-time RT-PCR revealed that A-83-01 blocked down-regulation of the mRNA expressions of *sox9*, *runx2*, and *osterix* by activin-A (Fig. 3C). To assess the effect of targeting activin-A on chondrocyte differentiation, we determined the effect of activin-A on cartilaginous matrix accumulation *in vitro*. Activin-A blocked the matrix accumulation of ATDC5, whereas A-83-01 prevented its inhibitory activity (Fig. 3D).

3.4. Effects of chemical inhibitors of signaling pathways on activin-A dependent suppression of chondrogenesis

To further elucidate the participation of the signaling molecules in response to activin-A, ATDC5 cells were treated with chemical inhibitors of the signaling pathway. Following treatment with SP600125 and LY294002, a substantial inhibition of JNK and Akt phosphorylation was observed (Fig. 4A). Both inhibitors down-regulated the expression of *sox9* mRNA (Fig. 4B, C). However, inhibitors of ERK (U0126) did not change *sox9* mRNA expression (data not shown).

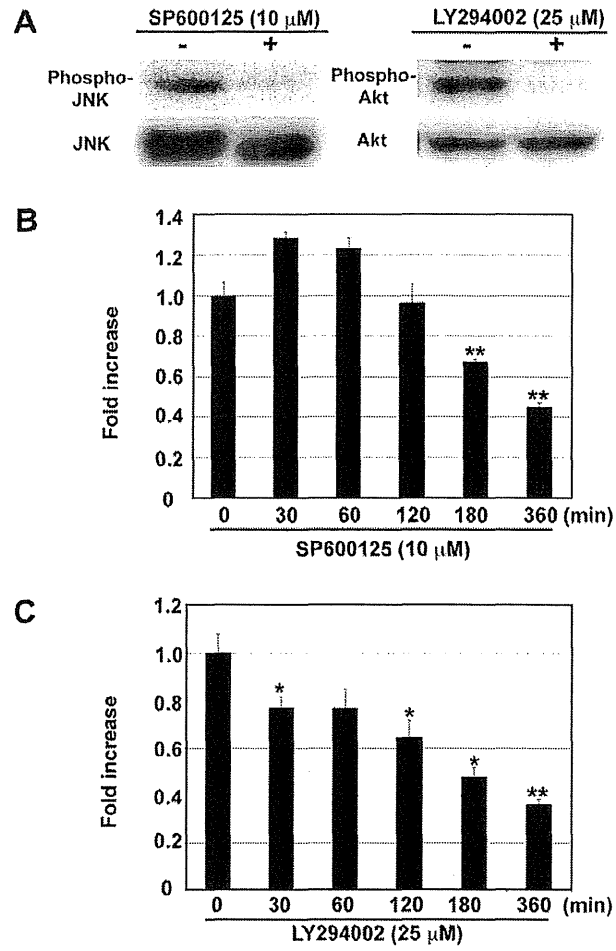

Fig. 4. Inhibitors of JNK and PI3K-Akt pathways suppress *sox9* mRNA expression. ATDC5 cells were incubated with SP600125 (10 μM) or LY294002 (10 μM) for the indicated times. (A) Whole cell lysates were probed using antibodies specific for phosphorylated JNK and Akt. Following visualization, the western blots were stripped and re-probed for JNK and Akt. (B, C) Total RNA was isolated and reverse-transcribed into cDNA using PCR amplification with SYBR green. PCR amplification was performed using *sox9*, and *gapdh*. Fold changes in *sox9* mRNA copy number values shown represent the average ± SD of data derived from triplicate cultures. $^{*}P < 0.05$; $^{**}P < 0.01$, by Student's *t*-test.

4. Discussion

In the present study, we used a clonal population of murine chondrogenic ATDC5 cells to elucidate the direct effect of activin-A on chondrocyte differentiation. ATDC5 cells were originally isolated from a differentiating culture of AT805 murine feeder-independent teratocarcinoma stem cells that expressed a fibroblastic cell phenotype in the growth phase on the basis of chondrogenic potential [20]. This cell line is known to be a useful *in vitro* model for examining chondrogenic differentiation [21,22] and endochondral ossification [21,23,24]. The main advantage of this system is that it does not contain any osteoblast/bone marrow stromal cells, which may also be targets of activin-A activity. We focused on chondro-progenitor cells to examine the effects of activin-A on differentiation and proteoglycan synthesis.

We found that activin-A suppressed chondrogenesis-related gene expression, including expressions of *sox9*, *runx2*, and *osterix in vitro*, while blocking activin-A signaling stimulated chondrocyte differentiation (Fig. 1). These findings are consistent with reports showing that activin-A acts as an inhibitor of chondrogenic differentiation in limb bud mesodermal cells [25], while they are in contrast with other studies showing that activin-A slightly stimulates proteoglycan synthesis in bovine articular cartilage [26]. We have no explanation for the reason of the discrepancy, though it may be due to differences in the cell lines used in these studies. Interestingly, we found that A-83-01 alone increased proteoglycan synthesis of ATDC5 (Fig. 3D). One explanation for this observation is that this agent binds activin-A, which endogenously enhanced proteoglycan synthesis.

TGF-β has been widely proven to regulate cell differentiation by triggering a large variety of signaling cascades, including the Smad family, and is tightly controlled by feedback mechanisms at different levels. Among them, activation of Ras, ERK1/2, and JNK by TGF-β signaling have been reported in primary intestinal epithelial cells and some breast cancer cell lines [27,28]. In addition, TGF-β activated kinase-1 (TAK1), a member of the MEKK family and activator of JNK and p38 MAPK pathways [29,30], is rapidly activated by TGF-β in certain cell systems, notably in murine C2C12 monocytes [31]. In the present study, we demonstrated that Smad2/3 and, ERK1/2 pathways were rapidly and transiently activated following

treatment with activin-A in ATDC5. Conversely, activin-A treatment caused a transient suppression of JNK and Akt phosphorylation (Fig. 2). Inhibition of Smad2/3 phosphorylation by type I TGF-β receptor kinase inhibitor revealed that these alternations of ERK1/2, JNK, and PI3K-Akt pathways are required for Smad2/3 phosphorylation (Fig. 3).

OA is generally thought to result from matrix destruction due to production of proteinases caused by a cryptic stimulus. However, the molecular mechanisms of cartilage destruction in OA remain largely unknown. Previous studies have revealed that the endochondral ossification process plays a crucial role in OA development as well as in physiological skeletal growth [2,32,33]. This process starts with hypertrophic differentiation of chondrocytes characterized by type X collagen expression, followed by conversion of non-vascularized and hypotoxic cartilage tissue into highly vascular invasion [34]. Aberrant expression of key markers involved in skeletogenesis, including *sox9*, was observed in mouse OA models [35]. Furthermore, significantly elevated expressions of *runx2* and *osterix* were observed in bone samples obtained from OA patients [36]. Our results in the present study showed that activin-A reduced the gene expression of *sox9*, *runx2* and *osterix*, suggesting that the activin-A administration may be useful as pharmacotherapy for treatment of OA.

In conclusion, we found that activin-A inhibited chondrogenesis in ATDC5 cells via down-regulation of JNK and Akt phosphorylation, suggesting the possible involvement of activin-A in regulation of chondrogenesis in pathophysiological conditions *in vivo*.

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編集後記

わが国は超高齢化社会となり、歯科領域では、これまでの齲蝕や歯周疾患だけでなく、口腔機能の低下に関連した口腔乾燥症や摂食嚥下障害が増加してきている。

本研究事業は、高齢者におけるドライマウスの実態調査と標準的ケア指針の策定を目的に、平成２２年度から３年間の総合的な研究を進めてきたが、最終年度である本年度は、これまでの研究成果をもとに、初年度の対象者を縦断的に調査し、全身状態、栄養状態ならびにドライマウスの経時的変化を把握するとともに、さらにそのリスク要因に関する統計学的解析を行うことで詳細な検討を行った。本研究成果が、高齢者におけるドライマウスの軽減や改善につながるとともに、高齢者の口腔機能が十分に発揮され、QOLの向上に貢献できると考えられた。

最後になりましたが、本研究事業にご協力いただきました関係各位の皆様方、ならびにご助言をいただきました皆様方に深謝申し上げます。

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厚生労働科学研究費補助金
長寿科学総合研究事業

高齢者のドライマウスの実態調査及び標準的ケア指針の策定に関する研究
平成 24 年度総括・分担研究報告書

発行日　　平成２５年３月３１日

発行者　　研究代表者　柿木保明(九州歯科大学　教授)

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TEL(093)581-1119　FAX(093)592-2151
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