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# Oxidized LDL is a Chemoattractant for the Eosinophils and Neutrophils

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## 산화 저비중 리포 단백이 호산구와 호중구에 대한 화학주성

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**연구배경 :**
기관지에 리노 바이러스(rhinovirus) 감염은 기관지혈관 내피세포의 투과성을 증가시켜 저비중리포단백(LDL) 같은 혈장단백의 유입을 초래한다. 그런데 산화 저비중리포단백(oxidized LDL)은 단핵세포 대식식세포에서 IL-1, GM-CSF 분비를 유발하고 화학주성과 또한 CD11b/CD18 intergrin을 증가시키며 L-selectin 표현을 감소시킨다. 이러한 소견들은 산화 저비중리포단백이 proimflammatory 효과를 가진다는 것을 시사한다. 연구자들은 산화 저비중리포단백이 리노바이러스 감염시 기도에 과립구를 동원할것이라는 가설하에 산화 저비중리포단백에 의한 호중구와 호산구의 화학주성과 내피세포이동(transendothelial migration)에 대하여 연구하였다.

**방 법 :**
저비중리포단백을 20-24시간 동안 5mM $Cu_2SO_4$로 산화 시키고 conjugated diens 형성 방법으로 234nm에서 산화 정도를 확인하였다. 과립세포들의 화학주성측정은 $3\text{-}5\times10_5$ 세포들을 transwell 필터에 놓고 37℃, 5% $CO_2$ 1시간 항온배양후 이동한 세포들을 혈구계로 계산하였다. 과립세포들의 내피세포이동은 인체 미세폐

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혈관 내피세포(human pulmonary microvascular endothelial cell) 들을 transwell 필터에 배양후 호산구와 호중구를 화학주성물질과 함께 놓은 후 3시간 항온 배양후 이동한 세포들을 혈구계로 계산하였다.

**결 과 :**

산화 저비중리포단백은 호산구와 호중구에 화학주성이있고 화학주성정도는 저비중리포단백의 농도와 산화 정도에 비례하였다. 또한 산화 저비중리포단백은 과립구의 인체 미세폐혈관 내피세포이동을 농도에 비례하여 자극하였고 호중구가 호산구보다 낮은 농도의 산화 저비중리포단백에 예민하게 반응하였다.

**결 론 :**

리노바이러스 감염으로 혈관투과성 증가로 저비중리포단백의 유입과 산화를 유발하고 이 산화 저비중리포단백이 기관지 간질세포에 호중구와 호산구이동을 유발하는 한 기전이며 또한 이과립구들이 산화 저비중단백과 함께 기도 염증을 초래할 것으로 사료된다. (Tuberculosis and Respiratory Diseases 2001, 51 : 211-223)

Key words : Oxidized LDL, Granulocytes, Transendothelial migration.

## Introduction

Viral respiratory infections are common exacerabators of asthma in both children, in whom 85 % of asthma excerbations may be caused by viral infection[1], and in 44% of adults[2]. It is still not known why viruses cause an increase in asthma severity

Current data[3] suggests that viral infections coordinately activate epithelial cells, endothelial cells, and leukocytes to cause airway edema, obstruction, and increased responsiveness. The epithelial cell is the sentinal cell to initiate antiviral immune responses through the secretions of a broad array of cytokines, chemokines, and mediators. The early activation of epithelial cells and other resident airway cells stimulate changes in endothelial cell physiology.

Endothelial cells are likely to contribute to airway inflammation via their role in recruiting leukocytes by increased expression of an adhesion molecule in the lung during respiratory viral infections and an increase in vascular permeability early in the course of infections. Exudation of plasma proteins due to increased vascular permeability is a major contributor to nasal mucosal edema and rhinorrhea, two hallmarks of viral infections[4].

LDL(low density lipoprotein) is a major constituent of serum and accounts for 2-3% of normal serum proteins[3]. In its native state, LDL does not affect inflammation. Normally circulating native LDL seldom undergoes oxidation ; when this does occur, the oxidized form is quickly converted back to the native form by circulating scavengers[5].

In inflammation, rhinovirus(RV), either directly or via RV-induced cytokines, can activate infiltrating neutrophils[4]. If a massive activation of neutrophils by RV takes place, these activated cells may change non-oxidized LDL(nLDL) into oxidized LDL(oxLDL).

For example, oxLDL participates in vivo atherogenesis. In the presence of a high plasma level of native LDL, native LDL leaks into the subendothelial space and can be changed into oxLDL by endothelial cells, smooth muscle cells, macrophages and neutrophils by the generation of reactive oxygen metabolites. The oxLDL recruit circulating monocytes by chemotaxis and is rapidly taken up in its oxidized form. Uptake of oxLDL by macrophages leads to the generation

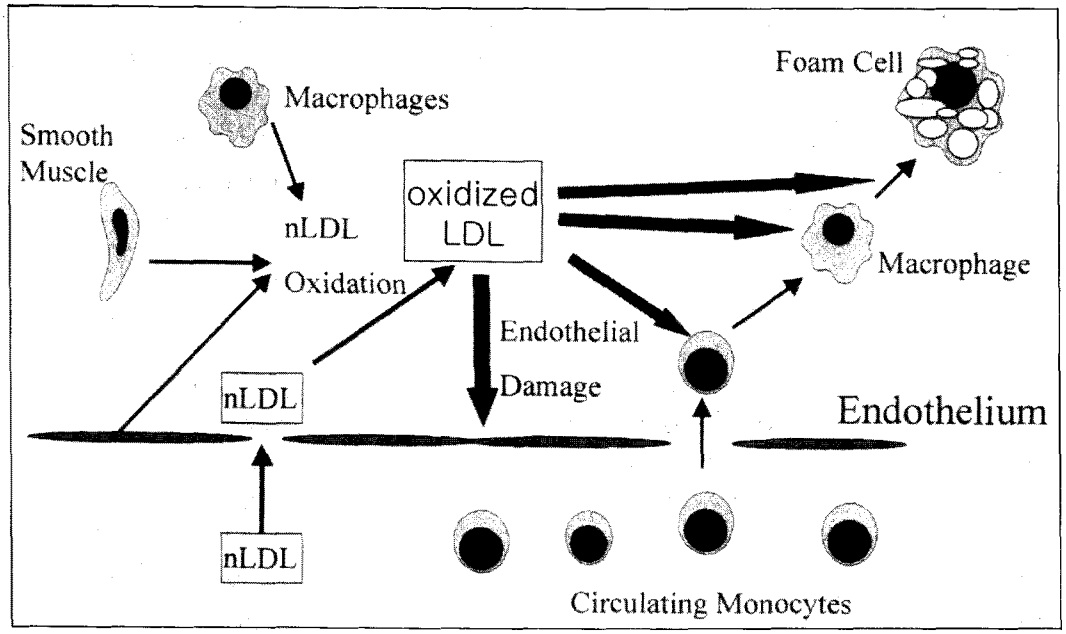


Fig. 1. Oxidation of nLDL in coronary artery.

of foam cells[5] (Fig. 1).

OxLDL has been demonstrated to exert cytotoxic effects at high concentrations[6]. However, at lower concentrations, OxLDL has proinflammatory properties including enhanced adhesion molecules of endothelial cells and monocytes[7], stimulation of IL-1 secretion by monocytes, differentiation of monocytes into macrophages, and the activation of T lymphocytes[8]. OxLDL induces enhanced INF-$\gamma$ secretion in lymphocytes from healthy individuals[9], and has been reported to be chemotatic for neutrophil and to stimulate neutrophil functions such as superoxide and adhesion[10]. However, little is known concerning the mechanisms by which oxLDL exerts these proimflammatory effects

We hypothesize that during common cold infections, increased vascular permeability and leakage of native LDL into the airways may result in LDL oxidation by activated airway cells leading in turn to increased inflammation and neutrophil/eosinphil recruitment. Moreover, this response is a possible mechanism for the early influx of granulocyte during viral infection. We studied the effect of oxLDL on neutrophil and eosinophil chemotaxis, transendothelial migration in vitro, a crucial cell in airway inflammation in asthma.

## Methods

### 1. Reagents and cytokines

Percoll was purchased from Pharmacia (Uppsala, Sweden). HBSS, RPML 1640 medium, PBS, newborn calf serum (NCS), trypsin EDTA, L-arginine, and penicillin -streptomycin were obtained from Life Technologies (Grand island,

NY, USA). Plasma fibronectin was obtained from Armour Pharmaceutical (Tuckahoe, NY, USA). Recombinant human IL-1β, PAF were purchased from R and D System (Minneapolis, MN, USA). Other reagents were purchased from (St. Louis, MO, USA) unless otherwise stated.

### 2. Human subjects

Eosinophils were isolated from the peripheral blood of subjects with allergic airway disease such as allergic rhinitis and mild asthma. Subjects ranged in age from 22 to 60 years, and the gender distribution was equal. Immediate hypersensitivity was confirmed by at least one positive skin reaction(>3mm), by the prick puncture technique, to extracts of common allergens, including ragweed, house dust mite, grass pollen, cat dander, and dog dander. Except for required inhaled β-agonists, subjects were taking no medications at the time of study. Informed consent was obtained before participation in the study, and the study was approved by the University of Wisconsin Human Committee.

### 3. Granulocyte seperation

Eosinophils(EOS) :

EOS were isolated using negative immunomagnetic bead selection, as previously described[11]. Briefly, heparinized blood was diluted with HBSS without $Ca^{2+}$ and centrifuged for 20min at 700xg over 1.090 g/ml percoll. Plasma, mononuclear cell band, and percoll were removed, and the RBCs in the pellet were lysed by hypotonic shock. The resulting granulocytes were washed with 4℃ HBSS supplemented with 2% NCS (HBSS/NCS). Concurrently, purified anti-CD16 from the mouse myeloma clone 3G8 (a generous gift from Dr. David M. Segal, National Cancer Institute, Bethesda, MD, USA) was incubated with goat anti-mouse-IgG-coated magnetic beads (PerSeptive Biosystems, Framingham, MA, USA) for 1 hour. The anti-CD16 bound beads were washed and incubated with granulocytes for 40 min in 4℃. Steel wool columns prepared in 10ml syringes were soaked for 2 to 4 hour with ehtanol, washed with HBSS, and filled with 4℃ HBSS/NCS. The cells and magnetic beads mixture was filltered through the column in a magnetic field (MACS system, Miltenyli Biotec, Aubum, CA, USA) to remove neutrophils bound to magnetic beads. CD16-negative EOS (>98% purity and >99%viability) were collected, washed, and suspended in enriched medium

Neutrophils :

A portion of the granulocytes (above) was set aside before anti-CD16 magnetic beads are added and designated as neutrophils. These cells were required to be >95% neutrophils and >95 % viable ; the containing cells were neutrophils. This enabled the study of both neutrophils and eosinophils from the same blood donor. Special care was taken to ensure that the granulocyte populations were not contaminated with mononuclear cells, particularly for the cytokine studies

### 4. Cell culture

Human pulmonary microvascular endothelial cell (HPMEC) cryopreserved as tertiary or quaternary cultures were purchased from Clonetics

(San Diego, CA, USA). These cells were isolated from the vasculature surrounding the alveolar sacs and were characterized as endothelial cells by Clonetics for acetylated low density lipoprotein uptake, and positive staining for platelet endothelial cell adhesion molecule (PECAM)-1 (CD-31) and Matrigel. Endothelial cell basal culture medium supplemented with 10ng/ml human recombinant epidermal growth factor, 1㎍/ml hydrocortizone ; 50㎍/ml gentamycin ; 50ng/ml amphotericin-B, 12㎍/ml bovine brain extract, and 5% PBS was obtained from Clonetics. To promote HPMEC attachment and growth, all culture surfaces were precoated with 10㎍/ml fibronectin for 1 hour at 37℃. Cells were passaged before they reached confluence. The profile of adhesion molecules on HPMEC has been determined to be equivalent between passages 3 and 12cells derived from two different donors were used at passages 5 through 9 in this study and found to give equivalent results[12].

### 5. Chemotaxis

Using Costar 24-well transwell plates, $3\text{-}5\times10^5$ cells were loaded into the transwell filter (3㎍ pore for neutrophils and 5㎍ for eosinophils, Costar, Cambridge, MA, USA) while the chemotatic agonist was placed in the lower well. The plates were incubated 1hour at 37℃, 5% $CO_2$. 10mM EDTA was added to the bottom wells to release any cells adherent to the underside of the filter and the number of migrating cells was counted in triplicate by hemocytometer and the percent migration was determined as (migrated eosinophil and neutrophil)/(total eosinophil and neutrophil added into upper compartment) × 100(%)

### 6. Transmigration

HPMEC ($2.5\times10^5$ cells/ml) were cultured on fibronectin coated transwell inserts (6.5 or 12mm diameter polycarbonate membrane with 8 ㎛ pores for eosinophil, 3㎛ pores for neutrophils, Costar, Cambridge. MA, USA). Medium was added into the upper compartment only to inhibit the formation of a HPMEC bilayer. HPMEC monolayers formed within 2 days and were confirmed for confluence and appreance appearance by Diff Quick staining(Baxter Scientific Products. Mcgaw Park. IL, USA). Both upper and lower compartments of the transwell were washed three times with 37℃ HBSS. Eosinophil and neutrophil ($3\text{-}5\times10^6$/ml) were then added in the lower compartment and the plates were gently vibrated to dislodge any migrated eosinophil and neutrophil, that were adherent to the bottom of the filter. Eosinophil and neutrophil were counted in triplicate by hemocytometer and the percent migration was determined as (migrated eosinophil and neutrophil)/(total eosinophil and neutrophil added into upper compartment) × 100(%)

### 7. Preparation of oxidized LDL

nLDL was obtained from Sigma. LDL was dialyzed with 6L of PBS for 3days to remove stabilizing EDTA and oxidized 5nM $Cu_2SO_4$ for 20-24 hours in a $CO_2$ incubator. Oxidation was arrested by the addition of 500㎛M EDTA.

Before use, both nLDL and oxLDL were

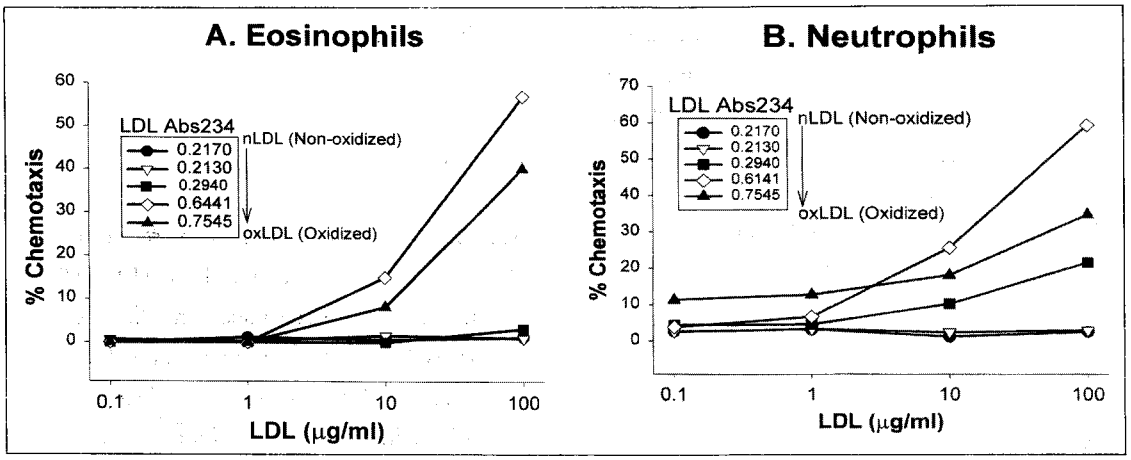


Fig. 2. Degree of LDL Oxidation vs Chemotaxis.

assessed for absorbance at 234mM to confirm their oxidative state by conjugated dienes formation[13]. Briefly, When LDL undergoes oxidation, it results in the formation of conjugated double bonds which have a characteristic absorption maximum at 234nm. The initial absorbance at 234nm was taken as the baseline and repeated every 10 minutes for 5 hours. The absorbance curve at 234nm is divided into 3 phases ; a lag phase, a propagation phase, and a decomposition phase. As long as the LDL is protected by antioxidants, the rate of increase at 234nm or the rate of formation of conjugated diens is very low (lag phase) ; upon, depletion of antioxidants, the rate is expected to increase in proportion to the initiating radicals (propagation phase) and when all the LDL oxidized, the rate is plateaus(decomposition phase)[14]. The oxidized LDLs were stored in a refrigerator(4℃) and were used for the experiment within 1 week.

### 8. Statistics

Data is presented as mean±SEM, and the groups were analyzed by ANOVA with repeated measures and Student's t test for paired comparisons. A p-value of less than 0.05 was considered significant.

## Results

### 1. Degree of LDL oxidation with chemotaxis

To determine the effect of oxidation of nLDL on the chemotaxis of eosinophils and neutrophils, nLDL at baseline and at increasing levels of oxidation (increasing Abs 234) were used as agonists. As the nLDL naturally oxidized, chemotaxis of both eosinophils and neutrophils increased, however the neutrophils were more sensitive to low levels of LDL oxidation than eosinophils. Interestingly, $Cu_2SO_4$-oxidized LDL (Abs234=0.7545) did not show a maximum stimulation of chemotaxis, possibly due to a cytotoxic effect on the cells(Fig. 2).

### 2. Chemotaxis

OxLDL, but not nLDL, is significantly chemotatic for eosinophils and neutrophils in 10-100μg/ml LDL concentrations($p<0.01$)(Fig. 3, 4). Both eosinophils and neutrophils underwent

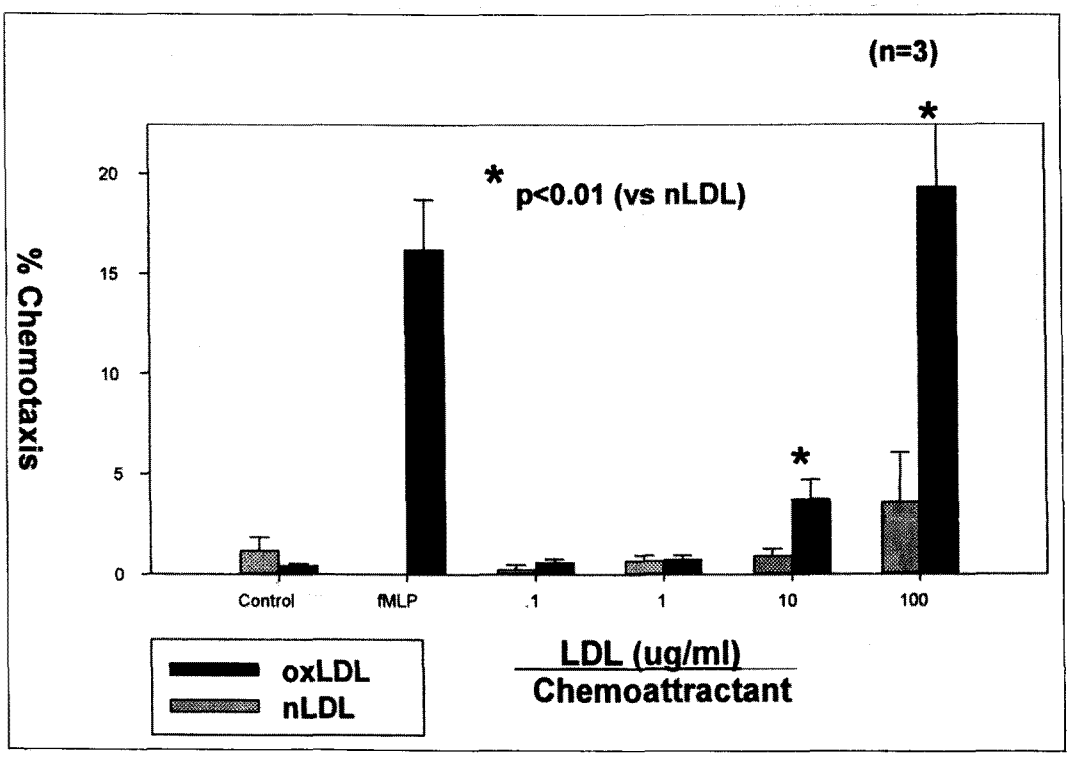


**Fig. 3.** Effect of LDL on Eosinophil chemotaxis.

chemotaxis to oxidized LDL in a concentration-dependent manner. Neutrophils were more sensitive than eosinophils in the 10 $\mu$g/ml LDL concentration ($p<0.05$). Chemotaxis of both neutrophil and eosinophil in 10-100 $\mu$g/ml LDLs concentrations were significantly higher than that of the control group($p<0.01$)(Fig. 5).

### 3. Transmigration

Pretreatment of HPMEC monolayers with IL-1$\beta$ increased granulocyte transmigration which reached significance in some conditions and again, granulocyte transmigration followed a concentration dependent rise(Fig. 6,7). Eosinophil transmigration through treated and untreated HPMEC monolayers in 100mg/ml LDL concentration were statistically higher than that of the control group ($p<0.05$)(Fig. 6). There was a significant difference in transmigration through the treated HPMEC between the control group and neutrophil in the greater than 1mg/ml LDL concentration($p<0.05$)(Fig. 7).

## Discussion

RV of the airways results in increased permeability of the airway vascular endothelium with the influx of plasma proteins, including lipids such as LDL[3]. OxLDL has been implicated in many proatherogenic events, while nLDL in the plasma has little proinflammatory function.

In vitro studies on the effect of oxLDL on leukocytes have demonstrated the induction of

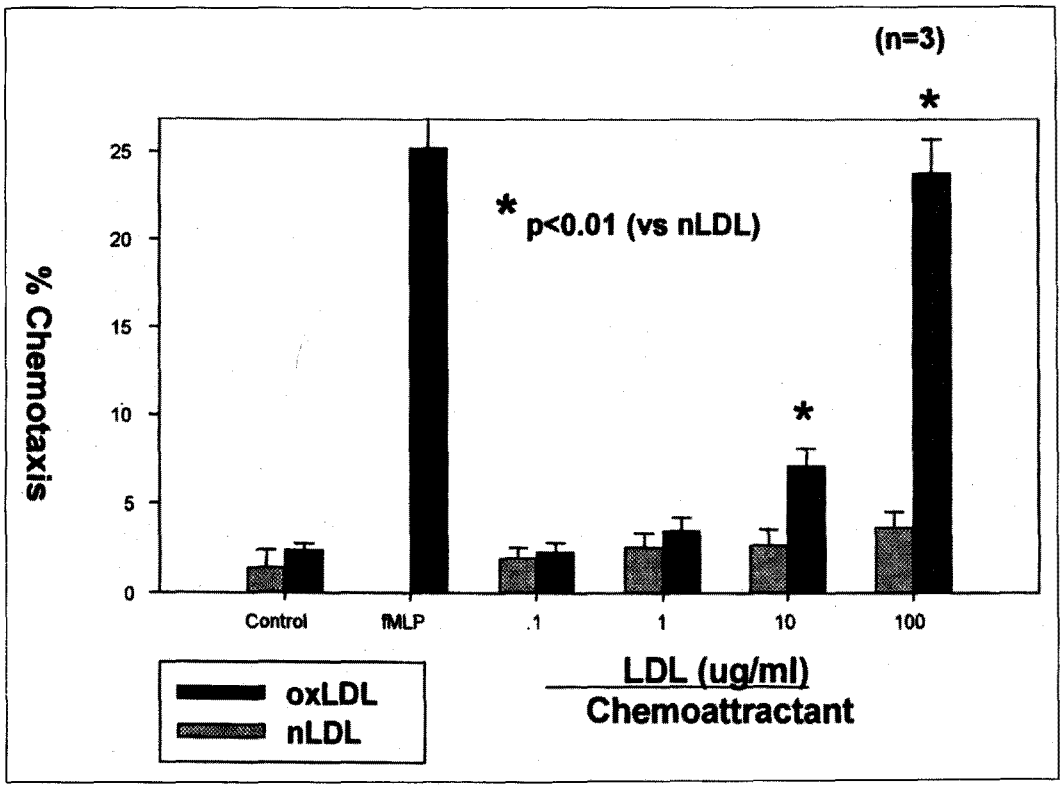


**Fig. 4.** Effect of LDL on neutrophil chemotaxis.

macrophage apoptosis as well as endothelial cell damage leading to increased vascular permeability at higher concentrations[6]. However, at lower levels, LDL oxidation can enhance endothelial cell and monocyte adhesion by increasing the expression of ICAM-1 and CD11b/CD18, respectively[7]. OxLDL can also induces IL-1 and GM-CSF secretion from monocytes/macrophages, increasing monocyte chemotaxis and promoting monocyte to macrophage differentiation[8]. It has recently been found that oxLDL activates and induces IFN-$\gamma$ secretion by T cells and a neutrophil respiratory burst, up regulation of CD11b/CD18 intergrins and decreases in L-selectin expression[9]. All of these findings suggest that oxLDL has many proinflammatory effects on leukocytes.

We hypothesized that oxLDL may be one mechanism of recruiting granulocytes to the airways during a RV infection. Therefore, chemotaxis and transmigration, in response to nLDL and oxLDL, were determined for these granulocytes.

This study has shown that oxLDL, not nLDL, is a potent chemoattrant for human neutrophils and eosinophils, depending on its degree of oxidation. OxLDL also stimulates eosinophil and neutrophil migration across HPMEC monolayers(+/- IL-1$\beta$ preactivation) in a dose dependent manner. However, neutrophils are more sensitive to low levels of oxLDL oxidation than eosinophils. This is the first study that has shown that

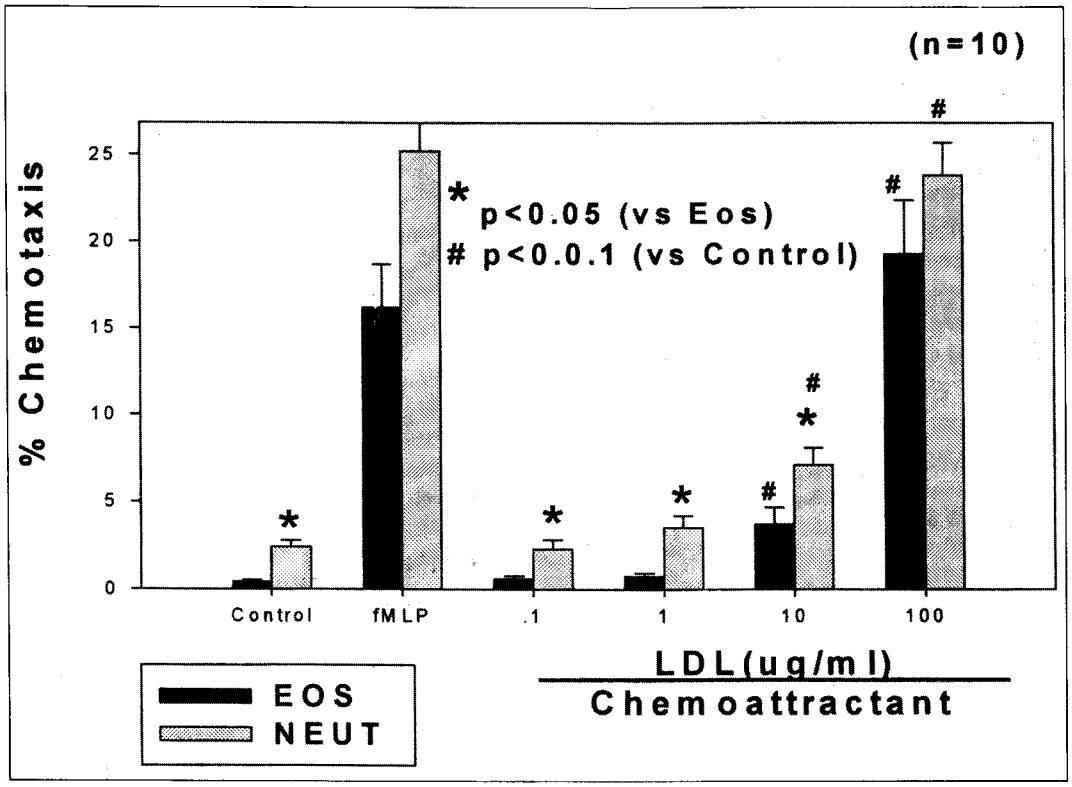


Fig. 5. Effect of oxLDL on granulocyte chemotaxis.

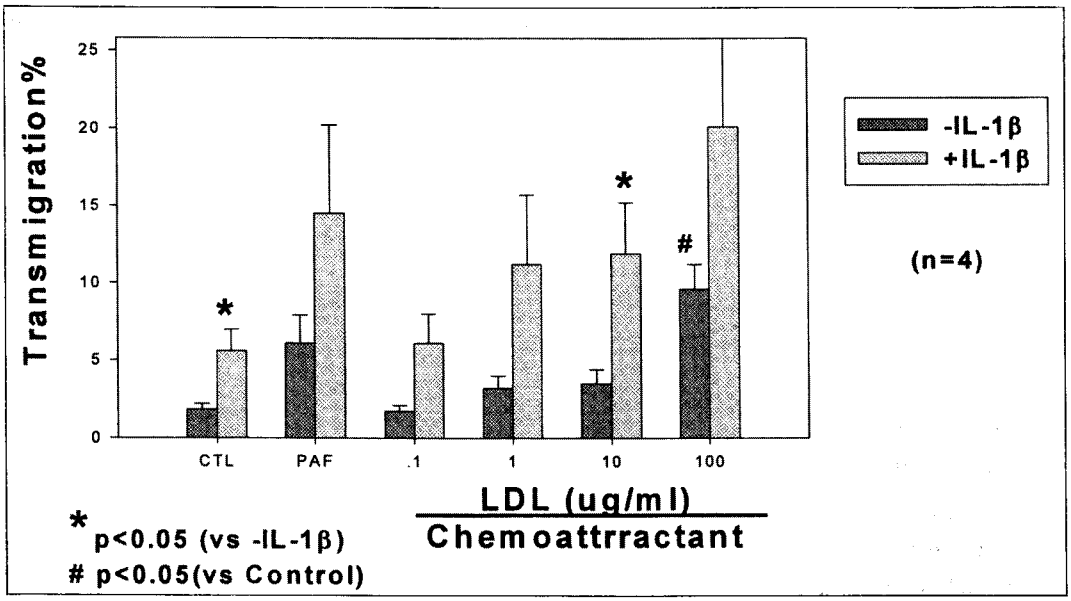


Fig. 6. Effect of oxLDL on eosinophil transmigration. (HPMEC +/- IL-1$\beta$)

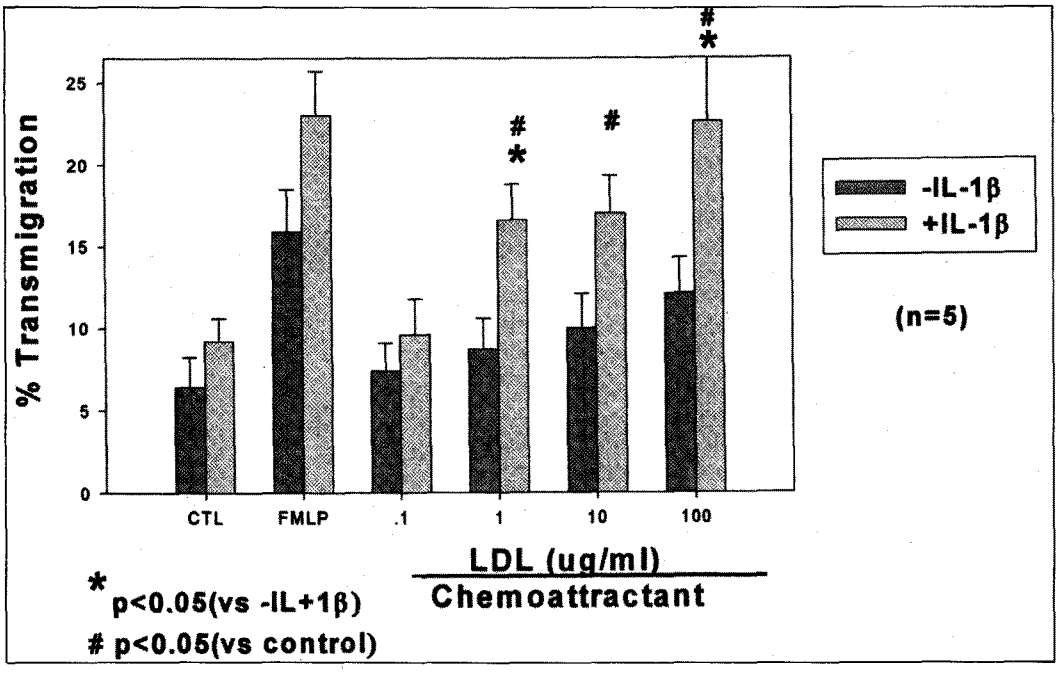


Fig. 7. Effect of oxLDL on neutrophil transmigration. (HPMEC +/- IL-1$\beta$)

oxLDL can affect eosinophil, a crutial cell in airway inflammation in asthma.

At a fundamental level, no one has yet been able to conclusively say where in the body the oxidation of LDL occurs. It is reasonably sure that it occurs in the artery wall, although it probably also occurs at sites of inflammation where LDL is exposed to activated macrophages and in other cells that have the ability to oxidize it, such as neutrophils[13]

Does the oxidation of LDL occur in the airway in vivo? In a normal physiologic condition, low amounts of oxLDL are produced and it may be cleared up by scavenger receptors. Yet if a massive activation of peripheral polymophonunuclear leukocytes by different agents takes place, these cells will excrete an enormous amount of oxLDL that can not be cleared out from the plasma. LDL undergoes oxidative modification following exposure to activated neutrophils[5]

In inflammation, rhinovirus either directly or via RV- induced cytokines can activate infiltrating neutrophils as well as resident macrophages and endothelial cells[4]. These activated cells generate oxygen metabolites which converts nLDL into oxLDL[10]

In turn, oxLDL can stimulate IL-8 production by endothelial cells as well as directly act as a chemoattractant for circulating neutorphils. Additionally, oxLDL increases CD11b/CD18 expression on speroxide generation by infiltrating neutrophil[15]. This may be one mechanism that begins neutrophil infiltration at the initiation of airway inflammation

What level of LDL is found in the airway?

The physiologic concentration of LDL leaking into the airways is unknown and is important in

order to compare with airway in vitro levels. In vitro, the concentration of oxLDL should be chosen to bracket quantities of oxLDL that may be present in the airway during respiratory viral infection. The peak concentration of albumin or IgG in nasal lavage fluid has been found to be 0.5-1.0% of the corresponding serum concentration[3].

The level of desirable serum LDL is less than 130mg/ml[16]. LDL in tissue can be effective at 0.5-2.0% of the serum level (0.65-2.6mg/ml) and the active dose of oxLDL in our experiments is 10-100ug/ml. We used a relevant low dose of oxLDL. Berliner et al[7] reported that exposure of endothelial cells to minimally modified LDL (as low as 0.12㎍/ml) enhances monocyte endothelial interaction. Therefore our experiments suggest oxLDL may reflect the level that can get to the airways through its effect on neutrophil and eosinophil inflammation. In order to determine LDL presence in the lung, it would be important to check LDL concentrations in the bronchoalveolar lavage, sputum and nasal washes during an RV infection and/or allergen challenge.

Which LDL receptor is effective in this LDL effects?

There are 3 LDL receptors : native LDL receptor, acetyl LDL receptor, oxidized LDL receptors[13]. The uptake of LDL by macrophages and by smooth muscles cells almost certainly does not occur by the native LDL receptor. We failed to obtain data on the blocking of chemotaxis and transmigration with nLDL. Acetyl LDL formed by treatment of LDL in vitro with acetyl anhydride is taken up much more avidly by macrophages. However there is no evidence that acetylated LDL occurs to any significant extent in vivo. There are at least five different macrophages receptors that can be involved in the binding and uptake of oxLDL. How important each of them may be under in vivo conditions remains to be established. It would be better to defer a blocking study until methods for assessing their function in vivo are developed and clarified.

In conclusion, increased vascular permeability during a RV infection may lead to the influx and oxidation of LDL. The resulting oxLDL. is one possible mechanism for the recruitment of neutrophils and eosinophils to the airway interstitial matrix. Once in the airways, granulocytes can further interact with oxLDL to promote airway inflammation.

## Summary

Background :
Rhinovirus infection of the airways results in increased permeability of the airway vascular endothelium with the influx of plasma proteins, including lipids such as LDL. In vitro studies on the effect of oxLDL on leukocytes has shown many proinflammatory effects on multiple leukocytes. We hypothesized that oxLDL is one mechanism for recruiting granulocytes to the airways during a RV infection. Therefore, chemotaxis and transendothelial migration , in response to nLDL, was determined for these granulocytes.
Methods :
nLDL was oxidized with 5mM Cu2S04 for 20-24 hours. 3-5 105 cells were loaded into the Transwell filter while the chemotatic agonists were placed in the lower well for chemotaxis. Confluent monolayers on HPMEC were grown on

Transwell filters for transendothelial migration. The filters were washed and eosinophils and neutrophils loaded on to the filter with the chemotatic agonist was were placed in the lower well. The wells were incubated for 3 hours. The number of migrating cells was counted on a hemocytometer

Results :

OxLDL, but not nLDL, is chemotatic for eosinophils and neutrophils. The level of granulocytes chemotaxis was dependent on both the concentration of LDL and its degree of oxidation. OxLDL stimulates eosinophil and neutrophils migration across HPMEC monolayers (+/- IL-1$\beta$ preactivation) in a dose dependent manner.

Conclusion

Increased vascular permeability during a RV infection may lead to the influx and oxidation of LDL. The resulting oxLDL. is one possible mechanism for the recruitment of neutrophils and eosinophils to the airway interstitial matrix. Once in the airways, granulocytes can further interact with oxLDL to promote airway inflammation.

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