40: 66—78, 1998.
29) Sakai H, Tomiyama K, Masada Y, et al: Pretreatment of serum containing Hb-vesicles (oxygen carriers) to avoid interference in clinical laboratory tests. Clin Chem Lab Med, 41: 222—231, 2003.
30) Wen D, Boissel JP, Tracy TE, et al: Erythropoietin structure-function relationships: high degree of sequence homology among mammals. Blood, 82: 1507—1516, 1993.
31) Braggins PE, Trakshel GM, Kutty RK, et al: Characterization of two heme oxygenase isoforms in rat spleen: comparison with the hematin-induced and constitutive isoforms of the liver. Biochem Biophys Res Commun, 141: 528—533, 1986.
32) Finch CA, Huebers H: Perspectives in iron metabolism. New Engl J Med, 306: 1520—1528, 1982.
33) Grady JK, Chen Y, Chasteen ND, et al: Hydroxyl radical production during oxidative deposition of iron in ferritin. J Biol Chem, 264: 20224—20229, 1989.
34) O'Connell MJ, Ward RJ, Baum H, et al: Iron release from haemosiderin and ferritin by therapeutic and physiological chelators. Biochem J, 260: 903—907, 1989.
35) Bennett GD, Kay MM: Homeostatic removal of senescent murine erythrocytes by splenic macrophages. Exp Hematol, 9: 297—307, 1981.
36) Ou LC, Kim D, Layton WM Jr., et al: Splenic erythropoiesis in polycythemic response of the rat to high-altitude exposure. J Appl Physiol, 48: 857—861, 1980.
37) Stutte HJ, Sakuma T, Falk S, et al: Splenic erythropoiesis in rats under hypoxic and post-hypoxic conditions. Virchows Arch A Pathol Anat Histopathol, 409: 251—261, 1986.
38) Okano K, Sogame Y, Ohkubo M, et al: Metabolic fate of recombinant human serum albumin (rHSA) (2). Jpn Pharmacol Ther (Yakuri tou Chiryou), 25 (Suppl): 2007—2018, 1987.
39) Baynes JW, Thorpe S: Identification of the sites of albumin catabolism in the rat. Arch Biochem Biophys, 206: 372—379, 1981.
40) Eckardt KU, Koury ST, Tan CC, et al: Distribution of erythropoietin producing cells in rat kidneys during hypoxic hypoxia. Kidney Int, 43: 815—823, 1993.
41) Hughes GS Jr, DeSmith VL, Locker PK, et al: Phlebotomy of 500 or 750 milliliters of whole blood followed by isovolemic hemodilution or autologous transfusion yields similar hemodynamic, hematologic, and biochemical effects. J Lab Clin Med, 123: 290—298, 1994.
42) Teramura Y, Kanazawa H, Sakai H, et al: Prolonged oxygen-carrying ability of hemoglobin vesicles by coencapsulation of catalase in vivo. Bioconjugate Chem, 14: 1171—1176, 2003.
43) Stuecklin-Utsch A, Hasan C, Bode U, et al. Pancreatic toxicity after liposomal amphotericin B. Mycoses, 45: 170—173, 2002.

ACUTE 40 PERCENT EXCHANGE-TRANSFUSION WITH HEMOGLOBIN-VESICLES (HBV) SUSPENDED IN RECOMBINANT HUMAN SERUM ALBUMIN SOLUTION: DEGRADATION OF HBV AND ERYTHROPOIESIS IN A RAT SPLEEN FOR 2 WEEKS

Hiromi Sakai[1], *Hirohisa Horinouchi*[2], *Manabu Yamamoto*[2], *Eiji Ikeda*[3], *Shinji Takeoka*[4], *Masuhiko Takaori*[5], *Eishun Tsuchida*[1] *and Koichi Kobayashi*[2]

[1]Advanced Research Institute for Science and Engineering, Waseda University
[2]Department of Surgery, School of Medicine, Keio University
[3]Department of Pathology, School of Medicine, Keio University
[4]Faculty of Science and Engineering, Waseda University
[5]East Takarazuka Sato Hospital

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Artificial Blood, Artificial Oxygen Carriers, Artificial Red Cells, Liposome, Plasma Substitutes

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Review

Hemoglobin Vesicle Aids Recovery of Cardiac Function during Ischemia-Reperfusion in Langendorff Perfused Rat Hearts

Tadashi Yamagishi, Motoaki Bessho, Miyuki Hama, Ryuichi Katoh,

Shigeki Yanagida, Masatoshi Kusuhara, Fumitaka Ohsuzu

Abstract

Background. - Formed from a lipid bilayer membrane, hemoglobin vesicle (HbV) is a small sphere (diameter ca. 250 nm) which contains hemoglobin, and is a candidate blood substitute. In this study, we examined whether HbV influences cardiac function during ischemia-reperfusion.

Methods. - Rat hearts were perfused according to the Langendorff method and subjected to 30 min of global ischemia and 30 min of reperfusion. HbV was made into a dispersion and diluted with Krebs-Henseleit buffer to achieve hemoglobin concentrations of 0.33 g/dL and 0.10 g/dL, and the hearts were perfused with this dispersion for 10 min immediately prior to ischemia. The same experiment was performed using the empty vesicle (EV; no hemoglobin). Cardiac functions were continuously monitored and coronary effluent collected every 5 min throughout the experiment.

Results. - In the HbV groups, between 10 and 30 min of reperfusion, there was a significant recovery in heart rate (to virtually pre-ischemia levels) as compared to the control group ($p< 0.05$). A rise in endodiastolic pressure during reperfusion was significantly suppressed in the HbV group (30-60 mmHg) as compared to the control group (70-100 mmHg) ($p< 0.05$). In the HbV groups, there was a significant recovery in left ventricular developed pressure (LVDP) between 20 and 30 min of reperfusion, as compared to the control group ($p< 0.05$). After 5 min of HbV perfusion and at 1 min of reperfusion, the lactate concentration in the coronary effluent was significantly lower in the HbV 0.33 g/dl group than in the control group ($p< 0.05$).

Conclusion. - These results suggest that HbV changed cardiac metabolism before and during ischemia, and as a result, enhanced recovery of cardiac function during reperfusion.

Keywords hemoglobin-vesicle, cardiac functions, lactate, ischemia-reperfusion

Introduction

Artificial red blood cells have the following features that reduce problems in clinical blood transfusions: (1) no need for time consuming cross-matching or typing, (2) no need for refrigeration, and (3) no potential as infectious agents. Therefore, artificial red blood cell substances have been widely investigated as potentially useful blood substitutes since the 1960s[1]. They include perfluorochemicals, various types of chemically modified hemoglobin, and recombinant human hemoglobin[2,3], and are more simple oxygen-carriers than artificial red blood cells. In 1993, Takeoka et al.[4] developed a method for the production of hemoglobin vesicles covered with a lipid bilayer membrane that could serve as artificial red blood cells. Since then, many studies on the functions[5,6] of hemoglobin vesicles as a blood substitute and their biological safety[7] have been conducted using various animal models. The data obtained in these studies suggested that hemoglobin vesicles had no serious adverse effects on cardiac functions in the models in which they were used but their actual effects on cardiac functions were not clarified. Therefore, in the present study, we examined whether HbV influences cardiac functions during ischemia-reperfusion using isolated rat hearts and the Langendorff perfusion technique. We found that HbV aids recovery of cardiac functions during ischemia-reperfusion in this model.

Materials and Methods

Hemoglobin-vesicle and empty-vesicle

Hemoglobin-vesicle (HbV), a small sphere[8] (diameter ca. 250 nm) formed from a lipid bilayer membrane which

Internal Medicine I, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan

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contains hemoglobin, is supplied by Oxygenix Co., Ltd. as a saline-suspension containing 10 g/dL of hemoglobin. Also supplied as a suspension, empty-vesicle (EV) is identical to HbV except that it does not contain hemoglobin. The HbV suspension was diluted with modified Krebs-Henseleit buffer to final hemoglobin concentrations of 0.33 g/dL (30 times dilution) and 0.10 g/dL (100 times dilution). The EV suspension was diluted in the same manner as HbV with modified Krebs-Henseleit buffer.

Animals and experimental groups

Eight-week-old male Wistar rats were purchased from Charles River Japan Inc. The rats were maintained under specific pathogen-free conditions and a constant dark/light cycle (12 h each) in our animal facility at the National Defense Medical College throughout the experiment. They were given free access to a laboratory chow CE-7 (Clea Japan, Tokyo) and water for a few weeks after purchase. A total of 33 rats were included in the present study and the experiments were performed when they were 9 to 12 weeks old. They were divided into five experimental groups: control group (n = 6), hemoglobin-vesicle 0.33 g/dL group (HbV 0.33 g/dL group, n = 6), hemoglobin-vesicle 0.10 g/dL group (HbV 0.10 g/dL group, n = 7), empty-vesicle 0.33 g/dL group (EV 0.33 g/dL group, n = 7), and empty-vesicle 0.10 g/dL group (EV 0.10 g/dL group, n = 7). All experiments were performed in accordance with the *National Defense Medical College Institutional Animal Care and Use Committee Guidelines.*

Heart preparation and perfusion method

The rats were pre-medicated with heparin (1,000U, i.p.), and 10 min later anesthetized with ketamine hydrochloride (90 mg/kg, i.p.) and xylazine hydrochloride (10 mg/kg, i.p.). The hearts were excised, put into ice-cold modified Krebs-Henseleit buffer (mKH buffer), quickly trimmed, weighed, and perfused according to the Langendorff mode (Fig. 1.). Perfusion was conducted at a constant perfusion pressure of 100 cmH_2O at 37°C with modified Krebs-Henseleit (mKH) buffer solution, which comprised NaCl 116 mM, KCl 4.7 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 25 mM, KH_2PO_4 1.2 mM, and glucose 11 mM. The experimental buffer solutions used (mKH buffer solution, two HbV containing mKH buffer solutions, two EV containing mKH buffer solutions) were continuously aerated with 95% O_2 + 5% CO_2 and the pH was adjusted to 7.4. Cardiac function was monitored and recorded using a fluid-filled left ventricular balloon in line with a transducer (P-50, Gould Inc.) and a WS-641G multi-channel recorder (Nihon Kohden, Tokyo, Japan). The balloon volume was set to produce a left ventricular end-diastolic pressure (LVEDP) of 0-5 mmHg.

In the control group, each heart was perfused with mKH buffer for 20 min (control perfusion) and then subjected to 30 min of global ischemia by stopping the perfusion. This was followed by 30 min of reperfusion. In the other experimental groups, each heart was subjected to perfusion with mKH buffer for first 10 min of the control perfusion and then perfusion with the respective vesicle containing buffer solution for the remaining 10 min. They were then subjected to 30 min of global ischemia and 30 min of reperfusion in the same manner as for the control group. To measure the coronary flow rate (CFR) and lactate content, the coronary effluent was collected at 5-min intervals during the control perfusion. During reperfusion, it was collected at 1 min and 4 min, and then at 5-min intervals until the end of experiment. After measuring the volume of the effluent, part of it was centrifuged at 10,000 x g for 40 min, and the supernatant was frozen and stored at -80°C until analysis for lactate content.

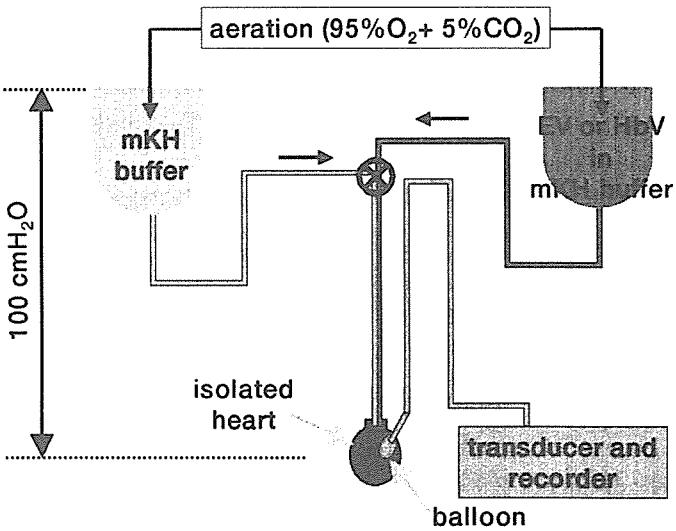

Fig. 1. Schematic presentation of Langendorff perfusion

Biochemical analysis and analysis of results

The coronary effluent was analyzed for lactate content enzymatically by the method of Lowry and Passonneau[9]. Cardiac function data [heart rate (HR), left ventricular endodiastolic pressure (LVEDP), and left ventricular developed pressure (LVDP)] were taken from the records made at 5-min intervals during the control perfusion and then at 10-min intervals until the end of experiment. In calculating the CFR for the 1st 5-min of reperfusion, the volume of the coronary effluents at 1-min and 4-min were added together and their sum taken as the volume for the 5-min interval. All values were calculated as mean ± SD but the SD has been

omitted to avoid confusion. All parameters were analyzed by means of time-series analysis of variance, and then the differences between the mean value in the control group and the corresponding values in the other experimental groups at each measuring time were analyzed using the Dunnett multiple comparison-test. A $p < 0.05$ was considered as significant.

Results

Cardiac functions

Coronary flow rate (CFR)

The mean CFR in the control group gradually decreased from about 17 mL/min to 15 mL/min during the control perfusion. Just after the onset of reperfusion, CFR started to increase. Between 5 and 10 min, it recovered to a level near that in the control perfusion period, and then showed a slight decrease until the end of reperfusion. With the change to mKH buffer containing HbV or EV for the last 10 min of the control perfusion in the respective groups, though there was a larger decrease in the mean CFR in the HbV 0.33 g/dL group and EV 0.33 g/dL group than in the control group, this difference was not significant. Changes in CFR in the reperfusion period were similar in the control and experimental groups (Fig. 2.).

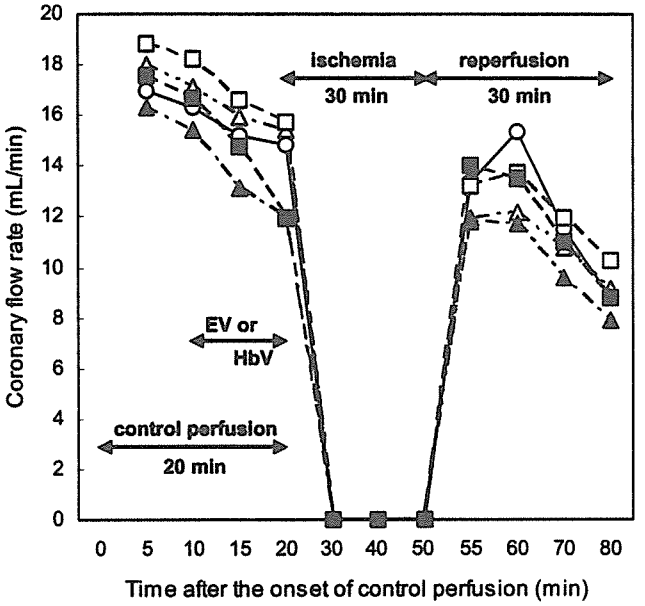

Fig. 2. Change in coronary flow rate (CFR) during experimental period
○: control group, □: HbV 0.10 g/dL group, ■: HbV 0.33 g/dL group, △: EV 0.10 g/dL group, ▲: EV 0.33 g/dL group

Heart rate (HR)

Mean HR values in the control group were maintained at about 260 beats/min during the control perfusion. HR was not affected by perfusing with mKH buffer solution containing HbV or EV for the last 10 min of the control perfusion. During the reperfusion period, there was no beating in any hearts of the control group apart from slight beating in one heart at 10 min. While the mean HR in the EV 0.10 g/dL group was similar to that in the control group, there was a slight recovery in HR in the EV 0.33 g/dL group, though this difference was not significant as compared to the mean HR in the control group. However, there was a significant recovery in HR in the two HbV groups (HbV 0.10 and 0.33 g/dL) ($p < 0.05$) compared with the control group at all measurement times during reperfusion. The mean HR in the HbV 0.33 g/dL group had recovered to the control perfusion level after 30 min of reperfusion (Fig. 3.).

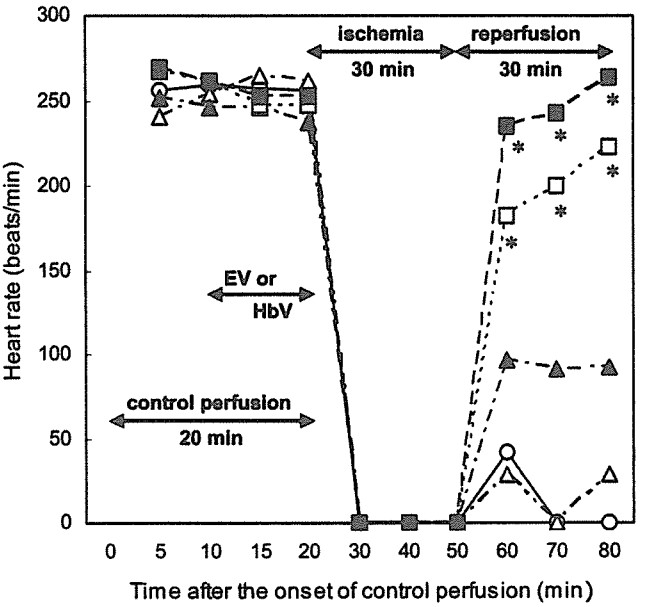

Fig. 3. Change in heart rate (HR) during experimental period
○: control group, □: HbV 0.10 g/dL group, ■: HbV 0.33 g/dL group, △: EV 0.10 g/dL group, ▲: EV 0.33 g/dL group
*$p < 0.05$, vs. control group by Dunnett multiple comparison test

Left ventricular endodiastolic pressure (LVEDP)

During the control perfusion, the mean LVEDP in the control group was maintained at about 5 mmHg. This value was not altered by perfusing with the mKH buffer solutions containing HbV or EV for the last 10 min of the control perfusion period. After the onset of ischemia, the mean LVEDP in the control group started to rise gradually, and at 30 min of ischemia it had reached about 40 mmHg. The changes in mean LVEDP in the two EV groups were similar

to that in the control group during ischemia. The mean LVEDPs in the 2 HbV groups seemed to have risen to a lesser extent than that in the control group at 30 min of ischemia, though this difference was not significant when compared with the control group. During reperfusion, the mean LVEDPs rose further to 80-90 mmHg in the control and EV 0.10 g/dL groups, and this rise was maintained until the end of reperfusion. In the EV 0.33 g/dL group, the LVEDP had risen to about 80 mmHg at 10 min of reperfusion and then decreased to about 65 mmHg at the end of reperfusion, though the latter LVEDP was not significant when compared with the corresponding value in the control group. In the 2 HbV groups, rises in the mean LVEDP (30-60 mmHg) were significantly ($p< 0.05$) suppressed as compared with the control group (70-100 mmHg) at 20 and 30 min of reperfusion (Fig. 4.).

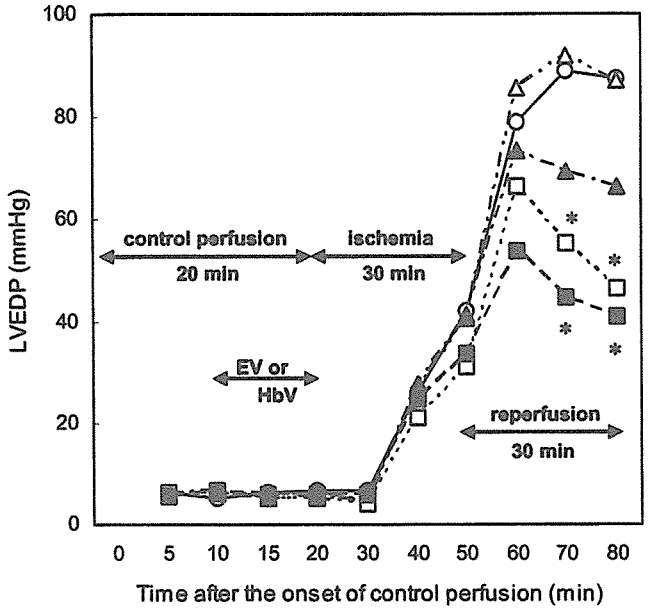

Fig. 4. Change in left ventricular endodiastolic pressure (LVEDP) during experimental period
○: control group, □: HbV 0.10 g/dL group, ■: HbV 0.33 g/dL group, △: EV 0.10 g/dL group, ▲: EV 0.33 g/dL group
*$p< 0.05$, vs. control group by Dunnett multiple comparison test

Left ventricular developed pressure (LVDP)

During the control perfusion, the mean LVDP in the control group gradually decreased from about 175 mmHg to 155 mmHg, and a similar decrease was observed in the other experimental groups. During reperfusion, apart from the development of a small amount of pressure in 1 heart at 10 min, no recovery in LVDP was observed in any heart in the control group, and the mean LVDPs in the EV 0.10 g/dL group were nearly the same as those in the control group at all measurement times. While there appeared to be a recovery in LVDP in the EV 0.33 g/dL group, this was not significant when compared with the control group. In contrast, at 20 and 30 min of reperfusion, there was a significant ($p< 0.05$) recovery in the mean LVDP in both HbV groups as compared with the control group (Fig. 5.).

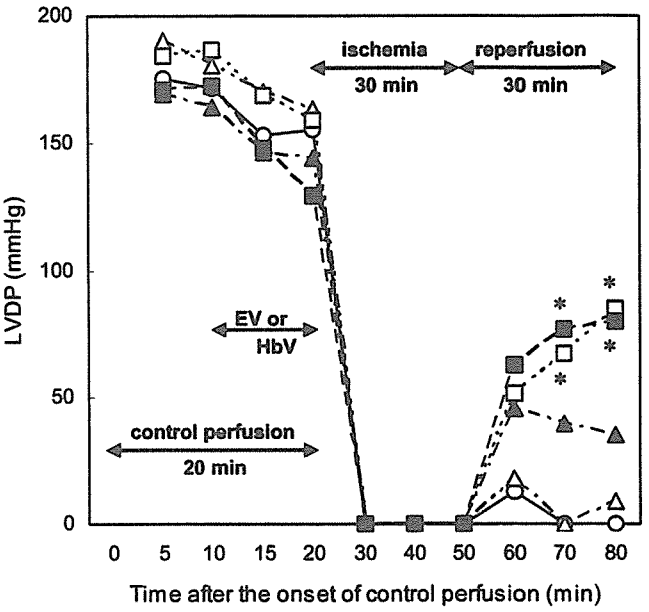

Fig. 5. Change in left ventricular developed pressure (LVDP) during experimental period
○: control group, □: HbV 0.10 g/dL group, ■: HbV 0.33 g/dL group, △: EV 0.10 g/dL group, ▲: EV 0.33 g/dL group
*$p< 0.05$, vs. control group by Dunnett multiple comparison test

Lactate concentration of coronary effluent

During the control perfusion, there was a gradual increase in the mean lactate concentration of the coronary effluent in the control group, from about 10 μg/mL to 18 μg/mL. Though the increase in mean lactate concentration seemed to be slightly suppressed for perfusion with mKH buffer containing EV 0.10 g/dL or 0.33 g/dL, the difference from the control group was not significant. In the HbV 0.10 g/dL and 0.33 g/dL groups, the mean lactate concentrations for 5 min after the onset of HbV perfusion were significantly ($p< 0.05$) lower than the corresponding control value (Fig. 6.A).

During reperfusion, there was a sharp increase in the lactate concentration of the coronary effluent in the control group in the first minute. In the next 4 min of reperfusion, it decreased rapidly to around the level in the control perfusion, and then continued at about this level until the end of reperfusion. Changes in the mean lactate concentration in the HbV 0.10 g/dL group were similar to those in the control group throughout the reperfusion period. The mean lactate

concentration in the HbV 0.33 g/dL group at 1 min of reperfusion was significantly ($p<0.05$) lower than that in the control group (Fig. 6.B). Though the mean lactate concentration in the HbV 0.33 g/dL group seemed to be slightly higher than that in the control group at 30 min of reperfusion, the difference was not significant.

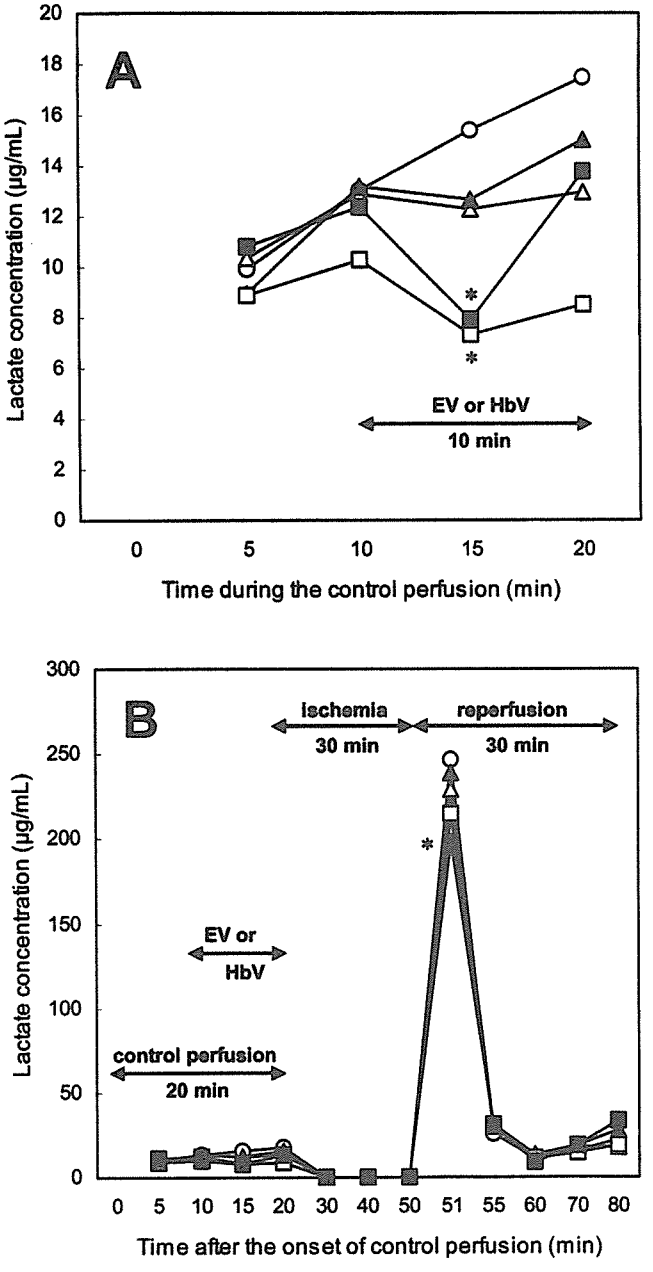

Fig. 6. Change in lactate concentration of coronary effluent during experimental period

A: Data obtained during control perfusion, B: Data obtained throughout the experiment. ○: control group, □: HbV 0.10 g/dL group, ■: HbV 0.33 g/dL group, △: EV 0.10 g/dL group, ▲: EV 0.33 g/dL group

*$p<0.05$, vs. control group by Dunnett multiple comparison test

Discussion

When an organ or a tissue is perfused with a buffer containing microcapsules like HbV and EV, we must be aware of the possibility of such microcapsules causing embolisms in the organ or tissue. Nakai et al.[10] reported that when isolated rat hearts were perfused with Krebs-Henseleit (KH) buffer containing hemoglobin-encapsulated liposomes (neo red cells, NRC), there was a sudden increase in perfusion pressure just after perfusion began, and their histological findings showed that embolisms were the likely cause of the increase in perfusion pressure. Further investigation revealed that inorganic crystals were formed after mixing NRC with the buffer, and these researchers suggested that the crystals were the cause of the embolisms. In this connection, Sakai et al.[11] showed that HbV caused no constriction of resistance arteries or hypertension in a conscious hamster model.

In the present study, we carefully observed cardiac functions while the hearts were perfused with mKH buffer containing HbV or EV for 10 min prior to ischemia. As mentioned in the results section, cardiac functions (CFR, HR, LVEDP, and LVDP) were not significantly affected by perfusing with mKH buffer containing HbV 0.33 g/dL or EV 0.33 g/dL. These results suggest that in contrast to the above research using NRC, embolisms did not occur in our study. Our findings with the higher concentrations of HbV and EV, however, require further clarification.

It is interesting that the lactate concentration of the coronary effluent was lower in the HbV 0.33 g/mL group than in the control group during the first 5 min of HbV perfusion and first minute of reperfusion. Pyruvate, the final substrate in the glycolytic pathway, is mainly oxidized in the mitochondria and partially converted to lactate by lactic dehydrogenase during control perfusion. Then, in ischemia, almost all pyruvate is converted to lactate since mitochondrial oxidation immediately stops, and this lactate is thought to be a factor in cardiac cell injury and delay in the recovery of cardiac functions during reperfusion. Therefore, we surmise that HbV suppressed lactate production in the cardiac cells during HbV perfusion and ischemia by stimulating mitochondrial oxidation, resulting in the significant recovery in cardiac functions in the HbV 0.33 g/dL group during reperfusion, though the mechanism by which HbV suppresses lactate production remains to be clarified.

In conclusion, our results suggest that HbV altered cardiac metabolism both before and during ischemia, and as a result, enhanced recovery of cardiac function during reperfusion.

Acknowledgements

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References

1. Chang TMS. New generations of red blood cell substitutes. J Intern Med 2003;253:527-535.
2. Squires JE. Artificial blood. Science 2002;8:295:1002-1005.
3. Winslow R. Current status of blood substitute research: Towards a new paradigm. J Intern Med 2003;253:508-517.
4. Takeoka S, Ohgushi T, Sakai H, Nishide H, Tsuchida E. Preparation conditions of human hemoglobin vesicles covered with lipid membrane. Artif Organs Today 1993;3:129-136.
5. Izumi Y, Sakai H, Hamada K, Takeoka S, Yamahata T, Kato R, Nishide H, Tsuchida E, Kobayashi K. Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carriers in anesthetized rats. Crit Care Med 1996;24:1869-1873.
6. Sakai H, Takeoka S, Wettstein R, Tsai AG, Intaglietta M, Tsuchida E. Systemic and microvascular responses to the hemorrhagic shock and resuscination with Hb-vesicles. Am J Physiol Heart Circ Physiol 2002;283:H1191-H1199.
7. Sakai H, Horinouchi H, Tomiyama K, Ideka E, Takeoka S, Kobayashi K, Tsuchida E. Hemoglobin-vesicles as oxygen carriers: Influence on phagocytic activity and histopathological changes in reticuloendothelial system. Am J Pathol 2001;159:1079-1088.
8. Sakai H, Hamada K, Takeoka S, Nishide H, Tsuchida E. Physical properties of hemoglobin vesicle as red cell substitutes. Biotechnol Progr 1996;12:119-125.
9. Lowry OH, Passonneau JV. In: A flexible system of enzymatic analysis. New York: Academic Press, 1972;194-196.
10. Nakai K, Usuba A, Ohta T, Kuwabara M, Nakazato Y, Motoki R, Takahashi TA. Coronary vascular bed perfusion with a polyethylene glycol-modified hemoglobin-encapsulated liposome, neo red cell, in rats. Artif. Organs 1998;22:320-325.
11. Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. Molecular dimensions of Hb-based O2 carriers determine constriction of resistance arteries and hypertension in conscious hamster model. Am J Physiol Heart Circ Physiol 2000;279:908-915.

Glucocorticoid Receptor Regulates ATP-Binding Cassette Transporter-A1 Expression and Apolipoprotein-Mediated Cholesterol Efflux From Macrophages

Makoto Ayaori, Shojiro Sawada, Atsushi Yonemura, Noriyuki Iwamoto, Masatsune Ogura, Nobukiyo Tanaka, Kazuhiro Nakaya, Masatoshi Kusuhara, Haruo Nakamura, Fumitaka Ohsuzu

Objective—The ATP-binding cassette transporter-A1 (ABCA1) regulates cholesterol efflux from cells and is involved in high-density lipoprotein metabolism and atherogenesis. The objective of this study was to investigate the effect of dexamethasone (Dex) and other glucocorticoid receptor (GR) ligands on apolipoprotein AI–mediated cholesterol efflux from macrophages and ABCA1 expression in them.

Methods and Results—Dex, a GR agonist, decreased ABCA1 mRNA levels in a dose- and time-dependent fashion, and RU486, a GR antagonist, reversed the inhibitory effect of Dex. The effects of Dex and RU486 on ABCA1 protein levels and apolipoprotein AI–mediated cholesterol efflux from the macrophages were consistent with these changes in mRNA levels. Transfected RAW264.7, together with a human ABCA1 promoter–luciferase construct, inhibited transcriptional activity by Dex and overexpression of human GR. Transrepression by GR was not mediated by liver X receptor (LXR), because there were no differences in the effects of the GR ligands on promoter activity between a reporter construct with mutations at the LXR binding site and one without the mutations, and no changes were brought about in ABCG1 and ABCG4 expression by GR ligands.

Conclusions—Our results showed that GR ligands affected ABCA1 expression and cholesterol efflux from macrophages, which are regulated by GR through a LXR-independent mechanism. (***Arterioscler Thromb Vasc Biol.* 2006;26:163-168.**)

Key Words: ABCA1 ■ dexamethasone ■ GR ■ cholesterol ■ macrophage

Multiple lines of evidence suggest that long-term use of glucocorticoids is associated with increased risk of developing atherosclerotic disease.[1] It has been reported that glucocorticoid treatment can cause cardiovascular complications in patients with rheumatoid arthritis[2] and systemic lupus erythematosus[3] receiving such treatment and that patients with Cushing's syndrome had a higher incidence of cardiovascular disease (CVD).[4] In this regard, it has been noted that glucocorticoid treatment causes hypertension, as well as impaired glucose and lipid metabolism,[1] which are classical risk factors for CVD. Other than this, glucocorticoids have been observed to directly affect the vascular wall with respect to cholesterol metabolism in the cells involved in atherogenesis.

Macrophage-derived foam cells containing accumulations of cholesteryl ester (CE) as lipid droplets are characteristically present in atherosclerotic plaque. In this connection, several investigators have reported that dexamethasone (Dex) induced CE accumulation in macrophages by enhancing scavenger receptor and acyl coenzyme A:cholesterol O–acyltransferase activity and reducing cholesterol esterase activity.[5,6]

Cholesterol efflux is a pivotal event in maintaining intracellular cholesterol levels and preventing the formation of macrophage-derived foam cells. Furthermore, the ATP-binding cassette transporter A1 (ABCA1) has been identified as a defective gene in Tangier disease,[7–9] which is characterized by an extremely low level of circulating high-density lipoprotein (HDL), and accumulation of CE in various tissues, which is concentrated in macrophage-derived foam cells. In addition, ABCA1 has been shown recently to play an important role in apolipoprotein AI (apoAI)–mediated cholesterol efflux from peripheral cells[10] and macrophages. Thus, given the key role of ABCA1 in facilitating cholesterol efflux, it would be of interest to determine how the ABCA1 gene itself is regulated. The expression of ABCA1 is greatly regulated by cAMP and sterols.[11–13]

Oxysterols and 9-*cis*-retinoic acid (9cRA) bind liver X receptor (LXR) and retinoid X receptor (RXR), respectively, to form heterodimers, which bind to conserved consensus

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From the First Department of Internal Medicine (M.A., S.S., A.Y., N.I., M.O., N.T., K.N., M.K., F.O.), National Defense Medical College, Tokorozawa, and the Mitsukoshi Health and Welfare Foundation (H.N.), Tokyo, Japan.

Correspondence to Makoto Ayaori, First Department of Internal Medicine, National Defense Medical College, 3-2 Namiki, Tokorozawa, Japan 359-8513. E-mail ayaori@ba2.so-net.ne.jp

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cis-element, direct repeat 4 (DR4), in the ABCA1 promoter region, resulting in the activation of transcription.[14,15] LXR, RXR, and the glucocorticoid receptor (GR) are all nuclear receptors that have been shown to play important physiological roles in macrophages.[16] Recent studies have demonstrated that the peroxisome proliferator–activated receptor-γ upregulates LXR expression resulting in elevated ABCA1 levels in macrophages[17] and that thyroid hormone receptor (TR) attenuated ABCA1 expression is dependent on LXR/RXR,[18] but it is still unclear whether it is glucocorticoids or GRs that influence cholesterol efflux and ABCA1 expression in macrophages.

In the present study, we found that the GR agonist Dex decreased apoAI-mediated cholesterol efflux and ABCA1 expression in macrophages, whereas the GR antagonist RU486 increased them, with ABCA1 expression in macrophages being regulated via GR.

Methods

The Methods section can be found in an online supplement available at http://atvb.ahajournals.org.

Results

Inhibitory Effect of GR Agonist Dex on ABCA1 Expression in THP-1 Macrophages and Restoration of Expression by GR Antagonist RU486

ABCA1 mRNA levels in the THP-1 macrophages decreased in a dose-dependent manner 6 hours after treatment with Dex and were restored by concomitant treatment with RU486, actually showing an increase compared with basal levels (Figure 1A). When the macrophages were treated with acetyl LDL (AcLDL) and the LXR/RXR agonists, 22HC and 9cRA, respectively, ABCA1 mRNA levels dramatically increased compared with basal levels. In this experiment, Dex attenuated ABCA1 mRNA levels, and RU486 restored them in the presence of AcLDL and the LXR and RXR agonists (Figure 1B and 1C).

Figure 1D shows that treatment with RU486 increased ABCA1 mRNA levels in a dose-dependent manner in the presence of untreated FBS but did not increase these levels in the presence of dextran-coated charcoal FBS. These findings indicate that increased ABCA1 expression because of RU486 stems from the endogenous glucocorticoid effect. We also investigated the effect of another GR agonist, triamcinolone, noting the attenuation of ABCA1 mRNA levels and antagonization by RU486 (Figure 1E). We also examined the changes in ABCA1 mRNA levels in THP-1 macrophages with time after treatment with Dex, finding that ABCA1 mRNA levels were slightly elevated after 2 hours and were then clearly lower at 4 hours and thereafter (Figure 2A). In the presence of untreated FBS, RU486 had increased ABCA1 mRNA levels after 2 hours, and the increase was maintained up to 6 hours (Figure 2B). Levels had decreased at 16 hours after treatment with RU486.

Effects of GR Ligands on ABCA1 Protein Levels and ApoAI-Mediated Cholesterol Efflux

Figure 3A shows the ABCA1 protein levels after treatment with Dex or RU486 in the presence and absence of AcLDL.

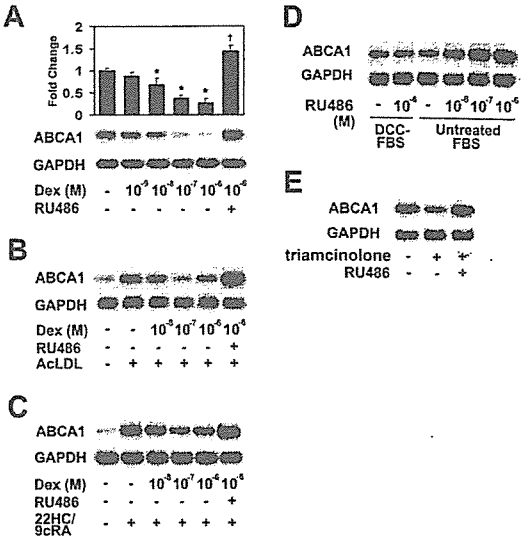


Figure 1. Effects of GR ligands on ABCA1 mRNA levels in THP-1 macrophages. After equilibration by incubation with the medium containing dextran-coated charcoal FBS (A through E) or untreated-FBS (D) for 24 hours, THP-1 macrophages were treated with vehicle, the indicated doses of Dex and RU486 (A through D), 1 μmol/L of RU486 (A through C and E), 50 μg/mL of AcLDL (B), 10 μmol/L of 22HC (C), 100 nM of 9cRA (C), and 1 μmol/L of triamcinolone (E) for 6 hours. Northern blot analysis was performed as described in Methods. For the graph in "A," the densitometry results from 3 separately performed experiments are presented as the mean±SE. *$P<0.05$ vs control; †$P<0.05$ vs Dex 1 μmol/L. *P* values were calculated using Mann–Whitney *U* test.

Dex decreased ABCA1 protein levels and RU486 restored them, even after stimulation of ABCA1 expression by AcLDL. We also examined the effects of the GR ligands on apoAI-mediated cholesterol efflux from THP-1 macrophages. Dex significantly reduced cholesterol efflux attributed to apoAI, and RU486 attenuated this inhibitory effect (Figure 3B), which was consistent with the changes in ABCA1 mRNA and protein levels.

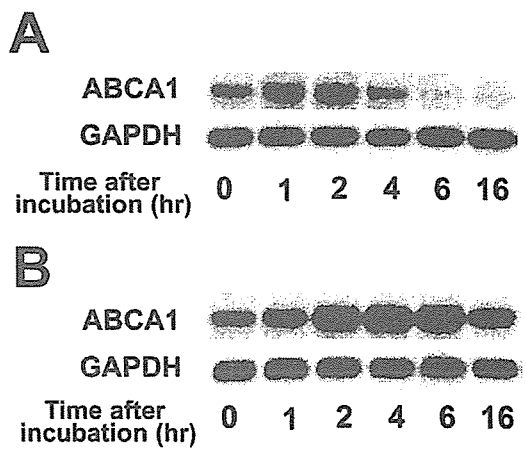


Figure 2. Changes in ABCA1 mRNA levels over time after treatment of THP-1 macrophages with GR ligands. A, Macrophages were treated with 1 μmol/L of Dex for the indicated periods. B, After equilibration by incubation with untreated FBS for 24 hours, macrophages were treated with 1 μmol/L of RU486 for the indicated periods. Northern blot analysis was performed as described in Methods.

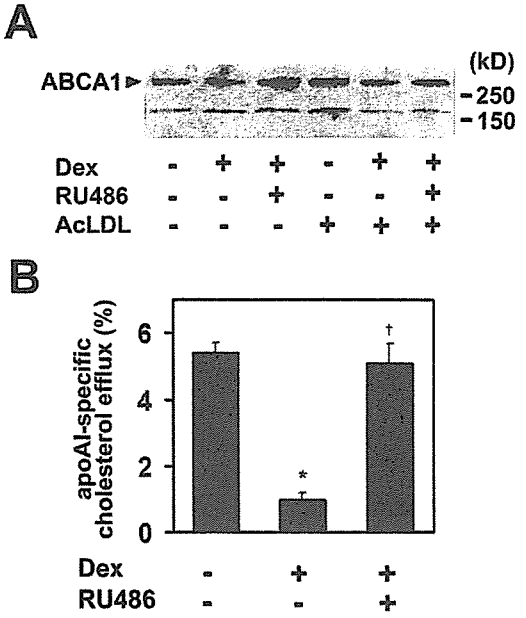


Figure 3. Effects of GR ligands on ABCA1 protein levels and apoAI-mediated cholesterol efflux from THP-1 macrophages. A, Sixteen hours after treatment with vehicle, 1 μmol/L of Dex, and RU486 in the presence or absence of 50 μg/mL of AcLDL, THP-1 macrophages were lysed and subjected to Western blot analysis as described in Methods. Upper bands are specific for ABCA1, and lower bands are nonspecific bands. B, Cholesterol efflux from macrophages mediated by apoAI. After cholesterol labeling, THP-1 macrophages were incubated with 1 μmol/L of the GR ligands or vehicle in the presence of 1 μg/mL of KW-3033 and 20 μg/mL of human apoAI for 16 hours. Cholesterol efflux was determined as described in Methods. For "B," the results for 4 samples are presented as the mean±SE. *P<0.05 vs control; †P<0.05 vs Dex 1 μmol/L. P values were calculated using Mann–Whitney U test.

Effects of GR Ligands on ABCA1 mRNA in HMDM and RAW264.7 Cells

We conducted experiments using human monocyte-derived macrophages (HMDM) and murine RAW264.7 cells to investigate whether Dex exerted inhibitory effects on ABCA1 and mRNA levels in other lines of the macrophages. By means of quantitative real-time RT-PCR, Dex was shown to significantly reduce ABCA1 mRNA levels in HMDM, and they were restored by RU486 (Figure IA, available online at http://atvb.ahajournals.org). In RAW 264.7 cells (Figure IB), Dex also reduced ABCA1 mRNA levels, and they were restored by RU486.

Regulation of ABCA1 Promoter Activity by GR Ligands through GR

To investigate whether GR ligands regulate ABCA1 mRNA levels in macrophages at the transcriptional level, a human ABCA1 promoter luciferase reporter construct spanning −940 to +110 bp (ABCA1-Luc) was transfected into RAW264.7 cells, and luciferase assay was performed. Dex inhibited ABCA1 promoter activity in a dose-dependent fashion, and RU486 completely restored it as shown in Figure 4A. In the case of overexpression of GR, ABCA1 promoter activity increased in the absence of Dex but decreased in its presence in a dose-dependent manner (Figure 4B). We also observed that when macrophages were treated with actinomycin D, the GR ligands had no effect on ABCA1 mRNA levels or mRNA stability (data not shown). These data suggest that GR ligands regulate ABCA1 expression in macrophages at the transcriptional level through GR.

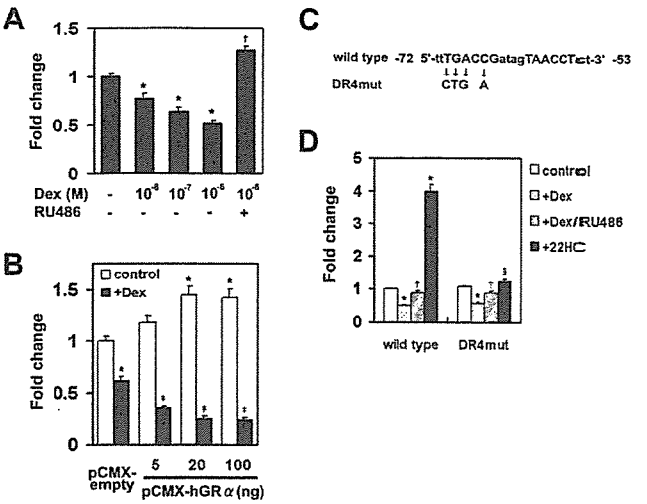


Figure 4. Effect of GR ligands on ABCA1 transcriptional activity in RAW264.7 cells. A, After transfection with ABCA1-Luc, the cells were treated with the indicated doses of Dex, 1 μmol/L of RU486, or vehicle. B, Effect of human GRα overexpression on promoter activity. The cells were treated with 1 μmol/L of Dex or vehicle. C, Mutated nucleotides in DR4mut construct. Numbers indicate relative positions to the transcription start site. Upper case indicates half-sites of DR4 element. D, Effect of mutation in DR4 on promoter activity. The cells were treated with vehicle, 1 μmol/L of GR ligands, or 10 μmol/L of 22HC. Luciferase assay was performed as described in Methods. For the graphs, the results for 4 samples are presented as the mean±SE. *P<0.05 vs control; †P<0.05 vs Dex 1 μmol/L; ‡P<0.05 vs control with pCMX empty vector; §P<0.05 vs wild-type with 22HC. P values were calculated using Mann–Whitney U test.

Alteration of ABCA1 Promoter Activity by GR Ligands Independent of LXR

To investigate whether the effects of GR ligands on ABCA1 promoter activity are associated with LXR- and RXR-dependent transcriptional regulation, we used a mutant reporter construct, DR4mut (Figure 4C). The LXR ligand 22HC failed to induce promoter activity for DR4mut, whereas it increased promoter activity in the case of the wild-type reporter construct ABCA1-Luc (Figure 4D). There were no differences in the changes in promoter activity because of GR ligands between ABCA1-Luc and DR4mut.

ABCG1 and ABCG4 are reportedly involved in cholesterol efflux from macrophages[19,20] and are also known to be LXR-responsive genes.[21–23] Figure IIA and IIB (available online at http://atvb.ahajournals.org) show that GR ligands did not affect mRNA levels of ABCG1 and ABCG4 in THP-1 macrophages, although they had been increased by LXR and RXR ligands. The above findings clearly demonstrate that transcriptional regulation in ABCA1 by GR ligands is independent of LXR. We also studied the effect of GR ligands on mRNA levels for scavenger receptor class B, type 1, which is reportedly involved in HDL-mediated cholesterol efflux,[24] but noted no change in levels (Figure IIC).

Discussion

We demonstrated that a GR agonist and antagonist, respectively, inhibited and stimulated ABCA1 expression in several lines of macrophages and apoAI-mediated cholesterol efflux from THP-1 macrophages, which were regulated through GR. Glucocorticoids have been reported to be associated with an increased risk of CVD presumably because of their unfavorable effects with respect to classical CVD risk factors, such as hypertension, impaired glucose, and lipid metabolism.[1] Other than these effects, glucocorticoids have been reported to directly affect the vascular wall with respect to cholesterol metabolism in the cells involved in atherogenesis. Hirsch and Mazzone[5] reported that Dex enhanced the degradation of AcLDL in macrophages through increased lipoprotein binding, which could mean enhanced scavenger receptor activity. Cheng et al[6] reported that Dex increased cholesterol O–acyltransferase activity and decreased neutral cholesterol esterase activity, resulting in greater accumulation of CE in macrophages. The homeostasis of intracellular cholesterol in peripheral cells is mainly maintained through its influx and efflux. Cholesterol efflux should be more important to macrophages than other types of cells, because macrophages express scavenger receptor class A, which is not downregulated by excess intracellular cholesterol. However, the effects of glucocorticoids on cholesterol efflux from macrophages have still to be investigated. Our study demonstrated the inhibitory effect of Dex on ABCA1 expression levels (Figures 1, 2, and 3A; Figure I) and apoAI-specific cholesterol efflux from macrophages (Figure 3B), which might be part of the reason for CE accumulation observed in previous studies. However, scavenger receptor class B, type 1 was not affected by the GR ligands, as mentioned above (Figure IIC). Petrichenko et al[25] reported that Dex decreased cholesterol efflux mediated by HDL from vascular smooth muscle cells, and Stein et al[26] observed that Dex decreased HDL-mediated cholesterol efflux from skeletal muscle in mice, suggesting that altered ABCA1 expression was involved.

It has been reported that glucocorticoid therapy increases serum HDL cholesterol levels.[27] Because GR is expressed in most organs, we investigated whether GR ligands affected ABCA1 expression in other types of cells. GR ligands did not change ABCA1 mRNA levels in NIH 3T3 cells, and Dex brought about a slight increase in levels in both HepG2 and Caco-2 cells, which was reversed by RU486 (data not shown). These results indicate that ABCA1 expression is regulated by GR ligands in a cell-specific manner, and partly explain the reason for the elevation of HDL cholesterol levels by glucocorticoids. Given that the contribution of ABCA1 in macrophages to serum HDL cholesterol levels is minimal, as reported by Haghpassand et al,[28] decreased ABCA1 expression in macrophages because of glucocorticoids is unlikely to affect HDL cholesterol levels.

GR agonists regulate gene expression in various ways, at the transcriptional,[29] posttranscriptional,[30–32] and posttranslational levels.[33] The classical mode of gene regulation by glucocorticoids, which accounts for most cases of positive gene regulation, is known to be mediated by interaction of ligand-activated GR with positive control elements, which are present in single or multiple copies upstream of or within target genes. However, compared with positive regulation of gene transcription by GR, negative regulation seems to be more complicated as described below. Dex reportedly destabilizes the mRNA of some genes presumably by affecting RNA binding proteins, which associate with 3′-untranslated lesions to stabilize mRNA.[30–32] Lasa et al[34] reported that Dex caused sustained expression of mitogen-activated protein kinase phosphatase 1 and phosphatase-mediated inactivation of mitogen-activated protein kinase p38, resulting in destabilization of Cox-2 mRNA. Kaplan et al[35] reported that a p38 inhibitor decreased ABCA1 expression in macrophages, and this inhibitory effect was not at the transcriptional level. In our study, the GR ligands did not affect ABCA1 mRNA levels or alter mRNA stability when macrophages were treated with actinomycin D (data not shown). Regarding transcriptional regulation, Dex inhibited ABCA1 promoter activity in a dose-dependent manner, and RU486 antagonized the inhibitory effects of Dex in this respect (Figure 4), which is consistent with the changes in mRNA levels observed. We also found that overexpression of GR decreased ABCA1 promoter activity in the presence of Dex. These findings indicate that GR ligands regulate ABCA1 expression in macrophages at the transcriptional level through GR.

We also observed a stimulatory effect of overexpression of GR in the absence of Dex (Figure 4B). Xiao and DeFranco[36] reported that overexpression of unliganded and liganded GR, respectively, activated and inactivated heat shock factor, a transcription factor that transactivates heat shock protein genes, such as hsp90 and hsp70. They suggested that the mechanism for increased transcriptional activity was unliganded GR-induced depletion of hsp70, which inactivates heat shock factor, resulting in activation of heat shock factor. In our study, overexpression of unliganded GRα might have affected unknown protein-regulated events involving ABCA1 expression, as Xiao and DeFranco[36] observed in the case of heat shock factor. Another possible mechanism is the reciprocal effects on transcriptional activity of liganded and unliganded receptors reported by Tagami et al.[37] They reported that transcription of genes, which was inhibited by liganded TR, was reciprocally activated by unliganded TR with enhancement by overexpression of nuclear corepressors. Because Xiao and DeFranco[36] clearly showed that unliganded GR was present not only in the cytosol but also in the nucleus, unliganded GR might stimulate ABCA1 promoter activity through the mechanism reported by Tagami et al.[37] Because we did not fully clarify the mechanism for the inhibitory effect of GR on ABCA1 transcription, it remains unclear whether activated GR is directly associated with the transcriptional machinery involved in ABCA1 expression. Given the cell-specific regulation of ABCA1 expression by GR ligands, GR might affect pathways recruited by macrophage-specific proteins associated with ABCA1 expression.

Dex treatment caused the transient increase in ABCA1 mRNA levels from 1 to 2 hours after beginning the experiment, and the levels gradually decreased from 4 hours onwards (Figure 2A). We also observed a difference in the times of maximal changes in ABCA1 expression between Dex and RU486, which were at 6 hours and 2 hours after treatment, respectively. The dependence on endogenous glu-

cocorticoids (Figure 1D) indicates that RU486 exerts a competitive effect on ABCA1 expression through GR antagonization. However, it is possible that different mechanisms are involved in the respective effects of Dex and RU486 and that they are the reason for the early and late times of maximal changes in ABCA1 expression. As described above, both liganded and unliganded GR reportedly affected not only gene transcription through binding control elements of target genes but also protein–protein interaction and intracellular protein distribution. Therefore, several mechanisms could be involved in the changes in ABCA1 expression attributed to GR ligands. Additional study will be needed to clarify the mechanism for the transient increase in ABCA1 expression because of Dex.

There are 2 main mechanisms for the negative regulation of gene transcription by GR, 1 of them involving interference with DNA binding of upstream or general transcription factors and the other a repression mechanism, which is independent of DNA binding. Ligand-activated GRs have been reported to interact with activator protein-1 (AP-1)[38] and nuclear factor κB (NF-κB),[39,40] resulting in decreased transcription of AP-1– and NF-κB–responsive genes involved in inflammation. The ABCA1 promoter has been found to have consensus sequences for AP-1 and NF-kB.[10,13] However, although GRs can affect ABCA1 promoter activity by interfering with AP-1 and NF-κB, Kaplan et al[35] reported that lipopolysaccharides induced ABCA1 expression in macrophages without activating transcription. Thus, additional studies will be needed to ascertain whether the inhibitory effect of GR on ABCA1 transcription is mediated though AP-1 and NF-κB.

Accumulating evidence indicates that LXR regulates many genes involved in cholesterol homeostasis in cells, such as ABCA1, ABCG1, and ABCG4. Recently, several investigators have demonstrated interactions between LXR and other nuclear receptors. TR has been reported recently to inhibit ABCA1 promoter activity through competition between TR/RXR and LXR/RXR heterodimers.[18] Small heterodimer partner, an unusual nuclear receptor, because it does not have the typical DNA binding domain, has been reported to act as a corepressor for several nuclear receptors, including LXR. Brendel et al[41] reported that small heterodimer partner attenuated ABCA1 and ABCG1 promoter activities by interacting with LXR. Our study revealed that GR-mediated transcriptional inhibition in ABCA1 is independent of LXR (Figure 4). In addition, the expression of other LXR target genes, ABCG1 and ABCG4, were not affected by GR ligands (Figure IIA and IIB).

Other recent studies have reported transcriptional regulation for both ABCA1 and ABCG1, not only by LXR and RXR and cholesterol loading, but also by zinc finger protein 202.[42] Moreover, Lorkowski et al[43] have reported that macrophages from patients with Tangier disease showed a compensated increase in ABCG1 expression. Although such evidence led us to believe that these 2 genes share similar mechanisms of regulation, we observed differences in the mechanisms of regulation of ABCA1 and ABCG1, and other researchers have also found this to be the case. Therefore, these genes may have their own unique functions, like that of ABCA1 in apoptosis.[44]

As other possible mechanisms for GR-mediated transrepression of ABCA1, there is the stimulation of ABCA1 expression in macrophages by transforming growth factor (TGF) β,[45] and inhibition of smad-dependent transactivation through the binding of smad3 by GRs.[46] To determine whether GR-mediated transrepression in ABCA1 is associated with the TGF-β signaling pathway, we investigated the effect of a neutral antibody against TGF-β on the changes in ABCA1 mRNA levels by GR agonists. The presence of the antibody, however, caused no changes in ABCA1 mRNA levels (data not shown).

Our study indicated that the attenuated cholesterol efflux and ABCA1 expression in macrophages could contribute to glucocorticoid-associated cardiovascular risk. Additional investigation of the mechanisms by which GR regulates ABCA1 expression would, therefore, provide important information for the study of compounds that facilitate cholesterol efflux.

References

1. Nashel DJ. Is atherosclerosis a complication of long-term corticosteroid treatment? *Am J Med.* 1986;80:925–929.
2. Kalbak K. Incidence of arteriosclerosis in patients with rheumatoid arthritis receiving long-term corticosteroid therapy. *Ann Rheum Dis.* 1972;31:196–200.
3. Salmon JE, Roman MJ. Accelerated atherosclerosis in systemic lupus erythematosus: implications for patient management. *Curr Opin Rheumatol.* 2001;13:341–344.
4. Etxabe J, Vazquez JA. Morbidity and mortality in Cushing's disease: an epidemiological approach. *Clin Endocrinol (Oxf).* 1994;40:479–484.
5. Hirsch LJ, Mazzone T. Dexamethasone modulates lipoprotein metabolism in cultured human monocyte-derived macrophages. Stimulation of scavenger receptor activity. *J Clin Invest.* 1986;77:485–490.
6. Cheng W, Kvilekval KV, Abumrad NA. Dexamethasone enhances accumulation of cholesteryl esters by human macrophages. *Am J Physiol.* 1995;269:E642–E648.
7. Brooks-Wilson A, Marcil M, Clee SM, Zhang LH, Roomp K, van Dam M, Yu L, Brewer C, Collins JA, Molhuizen HO, Loubser O, Ouelette BF, Fichter K, Ashbourne-Excoffon KJ, Sensen CW, Scherer S, Mott S, Denis M, Martindale D, Frohlich J, Morgan K, Koop B, Pimstone S, Kastelein JJ, Genest J Jr, Hayden MR. Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency. *Nat Genet.* 1999;22:336–345.
8. Bodzioch M, Orso E, Klucken J, Langmann T, Bottcher A, Diederich W, Drobnik W, Barlage S, Buchler C, Porsch-Ozcurumez M, Kaminski WE, Hahmann HW, Oette K, Rothe G, Aslanidis C, Lackner KJ, Schmitz G. The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. *Nat Genet.* 1999;22:347–351.
9. Rust S, Rosier M, Funke H, Real J, Amoura Z, Piette JC, Deleuze JF, Brewer HB, Duverger N, Denefle P, Assmann G. Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1. *Nat Genet.* 1999;22:352–355.
10. Wang N, Silver DL, Thiele C, Tall AR. ATP-binding cassette transporter A1 (ABCA1) functions as a cholesterol efflux regulatory protein. *J Biol Chem.* 2001;276:23742–23747.
11. Takahashi Y, Smith JD. Cholesterol efflux to apolipoprotein AI involves endocytosis and resecretion in a calcium-dependent pathway. *Proc Natl Acad Sci U S A.* 1999;96:11358–11363.
12. Oram JF, Lawn RM, Garvin MR, Wade DP. ABCA1 is the cAMP-inducible apolipoprotein receptor that mediates cholesterol secretion from macrophages. *J Biol Chem.* 2000;275:34508–34511.
13. Santamarina-Fojo S, Peterson K, Knapper C, Qiu Y, Freeman L, Cheng JF, Osorio J, Remaley A, Yang XP, Haudenschild C, Prades C, Chimini G, Blackmon E, Francois T, Duverger N, Rubin EM, Rosier M, Denefle P, Fredrickson DS, Brewer HB Jr. Complete genomic sequence of the human ABCA1 gene: analysis of the human and mouse ATP-binding cassette A promoter. *Proc Natl Acad Sci U S A.* 2000;97:7987–7992.

14. Costet P, Luo Y, Wang N, Tall AR. Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. *J Biol Chem.* 2000;275:28240–28245.
15. Repa JJ, Turley SD, Lobaccaro JA, Medina J, Li L, Lustig K, Shan B, Heyman RA, Dietschy JM, Mangelsdorf DJ. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. *Science.* 2000;289:1524–1529.
16. Valledor AF, Ricote M. Nuclear receptor signaling in macrophages. *Biochem Pharmacol.* 2004;67:201–212.
17. Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB, Liao D, Nagy L, Edwards PA, Curtiss LK, Evans RM, Tontonoz P. A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. *Mol Cell.* 2001;7:161–171.
18. Huuskonen J, Vishnu M, Pullinger CR, Fielding PE, Fielding CJ. Regulation of ATP-binding cassette transporter A1 transcription by thyroid hormone receptor. *Biochemistry.* 2004;43:1626–1632.
19. Klucken J, Buchler C, Orso E, Kaminski WE, Porsch-Ozcurumez M, Liebisch G, Kapinsky M, Diederich W, Drobnik W, Dean M, Allikmets R, Schmitz G. ABCG1 (ABC8), the human homolog of the Drosophila white gene, is a regulator of macrophage cholesterol and phospholipid transport. *Proc Natl Acad Sci U S A.* 2000;97:817–822.
20. Wang N, Lan D, Chen W, Matsuura F, Tall AR. ATP-binding cassette transporters G1 and G4 mediate cellular cholesterol efflux to high-density lipoproteins. *Proc Natl Acad Sci U S A.* 2004;101:9774–9779.
21. Venkateswaran A, Repa JJ, Lobaccaro JM, Bronson A, Mangelsdorf DJ, Edwards PA. Human white/murine ABC8 mRNA levels are highly induced in lipid-loaded macrophages. A transcriptional role for specific oxysterols. *J Biol Chem.* 2000;275:14700–14707.
22. Kennedy MA, Venkateswaran A, Tarr PT, Xenarios I, Kudoh J, Shimizu N, Edwards PA. Characterization of the human ABCG1 gene: liver X receptor activates an internal promoter that produces a novel transcript encoding an alternative form of the protein. *J Biol Chem.* 2001;276: 39438–39447.
23. Engel T, Lorkowski S, Lueken A, Rust S, Schluter B, Berger G, Cullen P, Assmann G. The human ABCG4 gene is regulated by oxysterols and retinoids in monocyte-derived macrophages. *Biochem Biophys Res Commun.* 2001;288:483–488.
24. Trigatti BL, Krieger M, Rigotti A. Influence of the HDL receptor SR-BI on lipoprotein metabolism and atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2003;23:1732–1738.
25. Petrichenko IE, Daret D, Kolpakova GV, Shakhov YA, Larrue J. Glucocorticoids stimulate cholesteryl ester formation in human smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 1997;17:1143–1151.
26. Stein O, Dabach Y, Hollander G, Ben-Naim M, Halperin G, Stein Y. Dexamethasone impairs cholesterol egress from a localized lipoprotein depot in vivo. *Atherosclerosis.* 1998;137:303–310.
27. Zimmerman J, Fainaru M, Eisenberg S. The effects of prednisone therapy on plasma lipoproteins and apolipoproteins: a prospective study. *Metabolism.* 1984;33:521–526.
28. Haghpassand M, Bourassa PA, Francone OL, Aiello RJ. Monocyte/macrophage expression of ABCA1 has minimal contribution to plasma HDL levels. *J Clin Invest.* 2001;108:1315–1320.
29. Adcock IM. Glucocorticoid-regulated transcription factors. *Pulm Pharmacol Ther.* 2001;14:211–219.
30. Korhonen R, Lahti A, Hamalainen M, Kankaanranta H, Moilanen E. Dexamethasone inhibits inducible nitric-oxide synthase expression and nitric oxide production by destabilizing mRNA in lipopolysaccharide-treated macrophages. *Mol Pharmacol.* 2002;62:698–704.
31. Garcia-Gras EA, Chi P, Thompson EA. Glucocorticoid-mediated destabilization of cyclin D3 mRNA involves RNA-protein interactions in the 3′-untranslated region of the mRNA. *J Biol Chem.* 2000;275: 22001–22008.
32. Dixon DA, Kaplan CD, McIntyre TM, Zimmerman GA, Prescott SM. Post-transcriptional control of cyclooxygenase-2 gene expression. The role of the 3′-untranslated region. *J Biol Chem.* 2000;275:11750–11757.
33. Kritsch KR, Murali S, Adamo ML, Ney DM. Dexamethasone decreases serum and liver IGF-I and maintains liver IGF-I mRNA in parenterally fed rats. *Am J Physiol Regul Integr Comp Physiol.* 2002;282:R528–36.
34. Lasa M, Abraham SM, Boucheron C, Saklatvala J, Clark AR. Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. *Mol Cell Biol.* 2002;22:7802–7811.
35. Kaplan R, Gan X, Menke JG, Wright SD, Cai TQ. Bacterial lipopolysaccharide induces expression of ABCA1 but not ABCG1 via an LXR-independent pathway. *J Lipid Res.* 2002;43:952–959.
36. Xiao N, DeFranco DB. Overexpression of unliganded steroid receptors activates endogenous heat shock factor. *Mol Endocrinol.* 1997;11: 1365–1374.
37. Tagami T, Madison LD, Nagaya T, Jameson JL. Nuclear receptor corepressors activate rather than suppress basal transcription of genes that are negatively regulated by thyroid hormone. *Mol Cell Biol.* 1997;17: 2642–2648.
38. Herrlich P. Cross-talk between glucocorticoid receptor and AP-1. *Oncogene.* 2001;20:2465–2475.
39. Almawi WY, Melemedjian OK. Negative regulation of nuclear factor-kappaB activation and function by glucocorticoids. *J Mol Endocrinol.* 2002;28:69–78.
40. McKay LI, Cidlowski JA. Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr Rev.* 1999;20:435–459.
41. Brendel C, Schoonjans K, Botrugno OA, Treuter E, Auwerx J. The small heterodimer partner interacts with the liver X receptor alpha and represses its transcriptional activity. *Mol Endocrinol.* 2002;16:2065–2076.
42. Porsch-Ozcurumez M, Langmann T, Heimerl S, Borsukova H, Kaminski WE, Drobnik W, Honer C, Schumacher C, Schmitz G. The zinc finger protein 202 (ZNF202) is a transcriptional repressor of ATP binding cassette transporter A1 (ABCA1) and ABCG1 gene expression and a modulator of cellular lipid efflux. *J Biol Chem.* 2001;276:12427–12433.
43. Lorkowski S, Kratz M, Wenner C, Schmidt R, Weitkamp B, Fobker M, Reinhardt J, Rauterberg J, Galinski EA, Cullen P. Expression of the ATP-binding cassette transporter gene ABCG1 (ABC8) in Tangier disease. *Biochem Biophys Res Commun.* 2001;283:821–830.
44. Hamon Y, Broccardo C, Chambenoit O, Luciani MF, Toti F, Chaslin S, Freyssinet JM, Devaux PF, McNeish J, Marguet D, Chimini G. ABC1 promotes engulfment of apoptotic cells and transbilayer redistribution of phosphatidylserine. *Nat Cell Biol.* 2000;2:399–406.
45. Argmann CA, Van Den Diepstraten CH, Sawyez CG, Edwards JY, Hegele RA, Wolfe BM, Huff MW. Transforming growth factor-beta1 inhibits macrophage cholesteryl ester accumulation induced by native and oxidized VLDL remnants. *Arterioscler Thromb Vasc Biol.* 2001;21: 2011–2018.
46. Song CZ, Tian X, Gelehuter TD. Glucocorticoid receptor inhibits transforming growth factor-beta signaling by directly targeting the transcriptional activation function of Smad3. *Proc Natl Acad Sci U S A.* 1999;96: 11776–11781.

High Plasma Levels of Osteopontin in Patients With Restenosis After Percutaneous Coronary Intervention

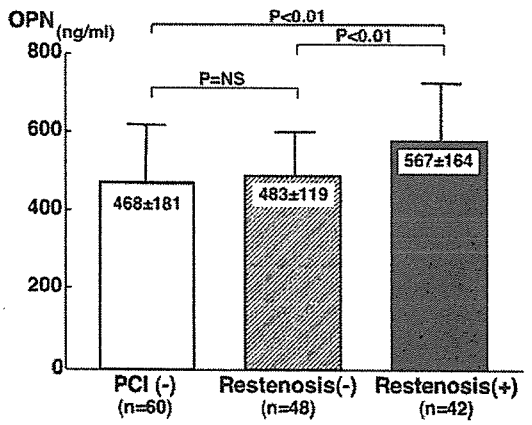
To the Editor:

High levels of osteopontin (OPN) mRNA and proteins were reported in atherosclerotic plaques.[1,2] Recently, we reported plasma OPN levels to be high in patients with coronary artery disease (CAD) and to correlate with the severity of CAD.[3] However, no association between plasma OPN levels and restenosis after percutaneous coronary intervention (PCI) has yet been demonstrated.

We measured plasma OPN levels in 90 patients with CAD undergoing elective coronary angiography for suspected restenosis. They had undergone PCI 0.6±0.4 years ago, of whom 52 (58%) had been treated with bare metal stents. OPN levels were also measured in 60 age- and gender-matched CAD patients with no history of PCI. Patients with acute coronary syndrome were excluded. Our study was approved by institutional ethics committee. After informed consent was obtained, fasting blood samples were taken. Plasma OPN levels were measured by ELISA (Human OPN assay kit; IBL), which measures total concentration of phosphorylated and nonphosphorylated forms of OPN. CAD was defined as at least one coronary artery having >50% luminar diameter stenosis. Restenosis was defined as >50% luminar diameter stenosis in the segment treated by PCI. Differences between 2 groups were evaluated by unpaired *t* test for parametric variables, by Mann–Whitney *U* test for nonparametric variables, and by χ^2 test for categorical variables. A probability value <0.05 was considered significant.

Of the 90 CAD patients with a history of PCI, 42 had restenosis. Compared with 48 CAD patients without restenosis, 42 with restenosis tended to have a higher rate of diabetes and a lower rate of smoking (Table). Among 3 groups, there was no difference in age, gender, or risk factors, except for total cholesterol levels. Plasma OPN levels were higher in CAD patients with restenosis than in those without restenosis and those with no history of PCI ($P<0.01$; Figure). CAD patients with restenosis more often had OPN level >600 ng/mL than those without restenosis and those with no history of PCI (38% versus 15% and 18%, $P<0.05$). OPN levels did not correlate with hsCRP, HbA1c, or fasting glucose levels. Clinical variables (age, gender, hypertension, hyperlipidemia, diabetes, smoking, stent, and hsCRP and OPN levels) were entered into multivariate logistic regression model. In addition to diabetes and smoking, OPN levels were independently associated with restenosis. Odds ratio for the presence of restenosis was 1.7 (95%CI=1.2 to 2.5; $P<0.01$) for a 100 ng/mL increase in OPN levels.

Restenosis after angioplasty is caused by negative arterial remodeling and neointimal proliferation, whereas in-stent restenosis is caused mainly by neointimal proliferation.[4] In vitro, OPN promotes the migration and proliferation of smooth muscle cells.[2] Increased OPN mRNA was shown in neointimal smooth muscle cells after arterial injury in animal models.[1] We generated OPN-overexpressing transgenic mice and demonstrated markedly increased neointimal formation after arterial injury.[5] Liaw et al[6] showed anti-osteopontin antibody treatment to reduce neointimal formation after injury in rat arteries. In humans, high levels of OPN mRNA and proteins were reported in atherectomy specimens from restenotic lesions.[7] Panda et al[2] showed plasma OPN levels in 13 patients undergoing coronary atherectomy to be elevated after atherectomy, and they remained high for at least 4 weeks. We showed plasma OPN levels to be higher in patients with than without restenosis and to be an independent factor for restenosis. OPN may play a role in the development of restenosis associated with neointimal proliferation.



Plasma OPN levels.

TABLE 1. Clinical Characteristics

	Restenosis (+) (n=42)	Restenosis (−) (n=48)	PCI (−) (n=60)
Age, years	65±10	61±9	63±9
Males	35 (83%)	43 (90%)	51 (85%)
Hypertension	27 (64%)	22 (46%)	38 (63%)
Systolic BP, mm Hg	129±14	127±17	135±20
Hyperlipidemia	28 (67%)	29 (60%)	35 (58%)
Total cholesterol, mg/dL	184±29	199±29	206±33*
HDL-cholesterol, mg/dL	48±14	49±14	47±11
Diabetes mellitus	16 (38%)	11 (23%)	14 (23%)
Current smoking	4 (10%)	10 (21%)	17 (28%)
Median hsCRP, mg/L	0.57	0.80	0.70
No. of >50% stenotic vessels	1.9±0.7	1.6±0.7	1.6±0.7
Multi-vessel disease	28 (67%)	24 (50%)	31 (52%)
Previous PCI procedures			
>1 lesions treated by PCI	2 (5%)	2 (4%)	
Stent placement	22 (52%)	30 (63%)	
Duration from PCI to angiography, years	0.6±0.3	0.7±0.4	
Current medication			
Statin	25 (60%)	23 (48%)	25 (42%)
ACE inhibitors or ARB	20 (48%)	25 (52%)	20 (33%)

Data are presented as mean±SD or the No. (%) of patients.

*$P<0.01$ compared with patients with restenosis (+).

Hypertension was defined as blood pressure ≥140/90 mm Hg or on drugs. Hyperlipidemia was defined as total cholesterol level >240 mg/dl or on drugs. Diabetes was defined as fasting glucose level ≥126 mg/dl or on hypoglycemic drugs or insulin.

ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blockers; hsCRP, high-sensitivity C-reactive protein.

Diabetes is a well-known clinical predictor of restenosis. In our study, diabetes was also a factor for restenosis. High glucose increases OPN mRNA in smooth muscle cells.[8] Increased OPN expression was shown in diabetic arteries.[9] Diabetes may thus facilitate restenosis via increased OPN production. However, OPN levels were a factor for restenosis independent of diabetes. Diabetes and increased OPN production may synergistically facilitate restenosis.

Our study was preliminary, because we did not measure OPN levels before PCI. Further study in a prospective manner is needed to elucidate the predictive value of plasma OPN levels before PCI for the development of restenosis. Moreover, we could not determine the main source of plasma OPN, because we did not measure OPN levels in coronary sinus.

Thus, high plasma levels of OPN in patients with a history of PCI were associated with the presence of restenosis, suggesting that OPN may play a role in the development of restenosis after PCI.

Ryuichi Kato
Yukihiko Momiyama
Reiko Ohmori
Nobukiyo Tanaka
Hiroaki Taniguchi
Koh Arakawa
Masatoshi Kusuhara
Haruo Nakamura
Fumitaka Ohsuzu
National Defense Medical College
Saitama, Japan

1. Giachelli CM, Bae N, Almeida M, Denhardt DT, Alpers CE, Schwartz SM. Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. *J Clin Invest*. 1993;92:1686–1696.
2. Panda D, Kundu GC, Lee BI, Peri A, Fohl D, Chackalaparampil I, Mukherjee BB, Li XD, Mukherjee DC, Seides S, Rosenberg J, Stark K, Mukherjee AB. Potential roles of osteopontin and $\alpha_v\beta_3$ integrin in the development of coronary artery restenosis after angioplasty. *Proc Natl Acad Sci U S A*. 1997;94:9308–9313.
3. Ohmori R, Momiyama Y, Taniguchi H, Takahashi R, Kusuhara M, Nakamura H, Ohsuzu F. Plasma osteopontin levels are associated with the presence and extent of coronary artery disease. *Atherosclerosis*. 2003; 170:333–337.
4. Schwartz RS, Henry TD. Pathophysiology of coronary artery restenosis. *Rev Cardiovasc Med*. 2003;3:S4–S9.
5. Isoda K, Nishikawa K, Kamezawa Y, Yoshida M, Kusuhara M, Moroi M, Tada N, Ohsuzu F. Osteopontin plays an important role in the development of medial thickening and neointimal formation. *Circ Res*. 2002; 91:77–82.
6. Liaw L, Lombardi DM, Almeida MM, Schwartz SM, deBlois D, Giachelli CM. Neutralizing antibodies directed against osteopontin inhibit rat carotid neointimal thickening after endothelial denudation. *Arterioscler Thromb Vasc Biol*. 1997;17:188–193.
7. O'Brien ER, Garvin MR, Stewart DK, Hinohara T, Simpson JB, Schwartz SM, Giachelli CM. Osteopontin is synthesized by macrophage, smooth muscle, and endothelial cells in primary and restenotic human coronary atherosclerotic plaques. *Arterioscler Thromb*. 1994;14:1648–1656.
8. Takemoto M, Yokote K, Yamazaki M, Ridall AL, Butler WT, Matsumoto T, Tamura K, Saito Y. Enhanced expression of osteopontin by high glucose in cultured rat aortic smooth muscle cells. *Biochem Biophys Res Commun*. 1999;258:722–726.
9. Takemoto M, Yokote K, Nishimura M, Shigematsu T, Hasegawa T, Kon S, Uede T, Matsumoto T, Saito Y, Mori S. Enhanced expression of osteopontin in human diabetic artery and analysis of its functional role in accelerated atherogenesis. *Arterioscler Thromb Vasc Biol*. 2000;20: 624–628.

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Review

The Effect of Interleukin-1 Receptor Antagonist on Arteries and Cholesterol Metabolism

Kikuo Isoda and Fumitaka Ohsuzu

Internal Medicine I, National Defense Medical College, Tokorozawa, Japan.

This review summarizes both the structure and function of IL-1 receptor antagonist (IL-1Ra), and relates our new findings, particularly those obtained in IL-1Ra-deficient mice (IL-1Ra$^{-/-}$), to the role of IL-1Ra in arterial diseases and cholesterol metabolism. IL-1Ra$^{-/-}$ mice show an increase in neointima-formation after arterial injury. Heterozygosity in the IL-1Ra gene against the apolipoprotein E-deficient background revealed a role for IL-1 in promoting atherogenic cell signaling and that the larger lesions of IL-1Ra$^{-/-}$ mice are enriched in macrophages and depleted of smooth muscle cells. Furthermore, IL-1Ra$^{-/-}$ mice developed severe fatty livers and hypercholesterolemia following 20 weeks on a atherogenic diet compared to WT mice. Taken together, these results suggest that IL-1Ra plays important roles in restenosis after angioplasty, the development of atherosclerosis, and the metabolism of cholesterol *in vivo*. *J Atheroscler Thromb, 2006; 13: 21–30.*

Key words: Interleukin-1 receptor antagonist, Inflammation, Atherogenesis, Cholesterol metabolism

Introduction

Interleukin (IL)-1 is a physiologically active factor produced and secreted by a variety of cells including those responsible for controlling immunity. Furthermore, it plays an important role in immune reactions, cell damage, and cell-proliferation (1, 2). IL-1 consists of two molecules, IL-1α and IL-1β, both of which exert similar but not completely overlapping biological functions mediated through the IL-1 type I receptor (IL-1RI). Another IL-1 receptor, the type II receptor (IL-1RII), has also been identified, but this receptor is not considered to be involved in signal transduction, but is rather thought to play a regulatory role as a "decoy". In addition, another member of the IL-1 gene family, IL-1 receptor antagonist (IL-1Ra), binds to IL-1 receptors without exerting agonistic activity. IL-1Ra as well as IL-1RII and the secretory forms of IL-1RI and IL-1RII are considered to negatively regulate IL-1 signaling (3).

The balance between IL-1 and IL-1Ra has significant effects on host responses to inflammation and infection (4, 5). In the immune system, IL-1 has many systemic effects in the protection of the body, being involved in fever, the response to stress, and the metabolism of insulin, lipid and bone (6, 7). Notably, in vascular homeostasis, IL-1 is considered one of the most potent proinflammatory cytokines acting on endothelial cells (ECs) and smooth muscle cells (SMCs) (8). IL-1 is produced from these cells as well as macrophages (Mφs) and hepatocytes (9, 10). IL-1 induces the expression of surface leukocyte adhesion molecules in ECs, proliferation of SMCs, and secretion of other cytokines and chemokines from ECs, SMCs, and Mφs (11, 12). These effects of IL-1 are strongly implicated in cardiovascular diseases (13–15). IL-1Ra, one of the negative regulators of IL-1 signaling, plays a role as an anti-inflammatory cytokine, similar to IL-10 and TGF-β, in acute- and chronic- inflammation of the vascular wall (16, 17). IL-1Ra is also produced by ECs and SMCs as well as Mφs for maintaining vascular homeostasis (18, 19). This re-

Address for correspondence: Kikuo Isoda, Internal Medicine I, National Defense Medical College, 3–2, Namiki, Tokorozawa, Saitama, 359–8513, Japan.
E-mail: isoda@me.ndmc.ac.jp
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view, focuses on the effects of IL-1Ra on atherogenesis and cholesterol metabolism.

Structure of Human IL-1 Ra

IL-1Ra was originally discovered as an inhibitor of IL-1 in the urine of patients with fever (20). A cDNA encoding the secreted form of the molecule was identified in a human monocyte library (21). Secretory IL-1Ra (sIL-1Ra) is synthesized as a 177-amino acid protein requiring the cleavage of a 25-amino acid leader sequence prior to secretion as a variably glycosylated 152-amino acid protein. A second cDNA coding for an intracellular form of IL-1Ra (icIL-1Ra) was cloned from a different human monocyte library (22). These two isoforms of IL-1Ra are created by alternative splicing yielding different first exons (23). The internal splice acceptor site for icIL-1Ra was located within the first exon for sIL-1Ra, near the 3' end of the sequence coding for the signal peptide. icIL-1Ra does not have a functional leader sequence and remains in the cytoplasm. The sIL-1Ra protein is produced by many cells that can synthesize IL-1. icIL-1Ra is found constitutively in keratinocytes and other epithelial cells but is also a delayed product of stimulated Mϕs (24, 25). Neutrophils contain only sIL-1Ra mRNA, whereas fibroblasts are capable of producing the mRNA and protein for both IL-1Ra isoforms, when appropriately stimulated (26).

Function of IL-1 Ra

In spite of extensive studies on IL-1 over the past two decades, the important roles that this cytokine may play in normal biology are unclear (27, 28). Furthermore, it remains unknown whether the function of IL-1Ra is limited to regulating the agonistic effects of extracellular IL-1 in normal biologic processes or in pathophysiological conditions. Studies on the functional consequences of overexpression or absence of expression of IL-1Ra in transgenic or knockout mice, respectively, may clarify some possible roles of this cytokine in normal biology. This review relates our new findings (obtained from IL-1Ra-deficient mice (IL-1Ra$^{-/-}$)) about the effects of IL-1Ra on arteries and cholesterol metabolism.

IL-1Ra and neointima formation after injury

Neointimal hyperplasia is characterized by the activation, migration, and proliferation of SMCs and is associated with inflammatory mediators such as cytokines. IL-1β is a chemoattractant and mitogen for SMCs (28) that is overexpressed at sites of the active proliferation and migration of this cell type subsequent to injury (29). Furthermore, a recent report demonstrated that IL-1RI gene-deficient mice tended to develop less neointima than wild-type mice (30). In sum, these previous studies suggested that IL-1 might promote neointimal formation. However, it remained uncertain whether IL-1Ra, the endogenous inhibitor of this central cytokine, could significantly suppress this response in the vasculature. Using IL-1Ra$^{-/-}$ mice (on the C57BL/6J background) and wild-type (IL-1Ra$^{+/+}$) mice, we investigated neointimal formation 3 weeks after femoral artery injury induced with an external vascular cuff. The mean intimal thickness and the intima/media ratio of IL-1Ra$^{-/-}$ mice increased by 249% and 257%, respectively, compared with IL-1Ra$^{+/+}$ mice (Fig. 1A) (31). Control immunostaining for IL-1Ra in injured vessels identified IL-1β and the endogenous inhibitor in the endothelium and inflammatory cells of adventitia in IL-1Ra$^{+/+}$ mice but not IL-1Ra$^{-/-}$ mice (Fig. 1B) (31). These results suggest that IL-1Ra protein prevents inflammation of both the intima and adventitia after cuff injury. Indeed, IL-1Ra$^{-/-}$ mice showed an increase in the proliferating cell nuclear antigen (PCNA) index of the intima and adventitia after injury. Within the adventitia, proliferating monocytes and macrophages comprised the majority of PCNA-positive cells. Recent studies have shown that adventitial passive fibroblasts can become active myofibroblasts under conditions of adventitial inflammation (32, 33). On the other hand, SMCs were the predominant proliferating cell type in the intima (31). IL-1 itself is a mitogen for SMCs (28), and furthermore, a recent study showed that vascular intima formation after mechanical injury mainly involves inflammatory cells that originate from the bone marrow (34). Our study demonstrated definitively that a deficiency of endogenous IL-1Ra promotes neointimal formation, revealing a crucial role for this protein in hyperplastic responses of the vasculature. Our results may be compatible with the report that p80 IL-1 type I receptor knockout mice tended to develop a smaller (7-fold) neointimal area induced by low shear stress compared to wild-type controls (30). This report demonstrated that IL-1 modulates low shear stress-induced neointimal formation, thus providing a direct proinflammatory cytokine signaling link between biomechanical forces to a vessel wall and the remodeling response of the artery. They also concluded that specific anti-IL-1 therapy may lessen neointimal formation.

IL-1Ra and atherogenesis

Atherogenesis is a complex process in which the activation of ECs and SMCs appears to be a central theme (8). IL-1 is produced by these cells as well as Mϕs and hepatocytes (9, 10). Furthermore, stimulation and activation of ECs and SMCs by IL-1 causes a wide range of inflammatory processes within the atheroma, such as the enhanced expression of leukocyte adhesion molecules (9, 12), clotting factors and inhibitors of fibrinolysis (11), and chemokines (28), as well as increased proliferation of SMCs (8, 15), suggesting a central role for IL-1 in the development of atherosclerosis. The activity of IL-1 is

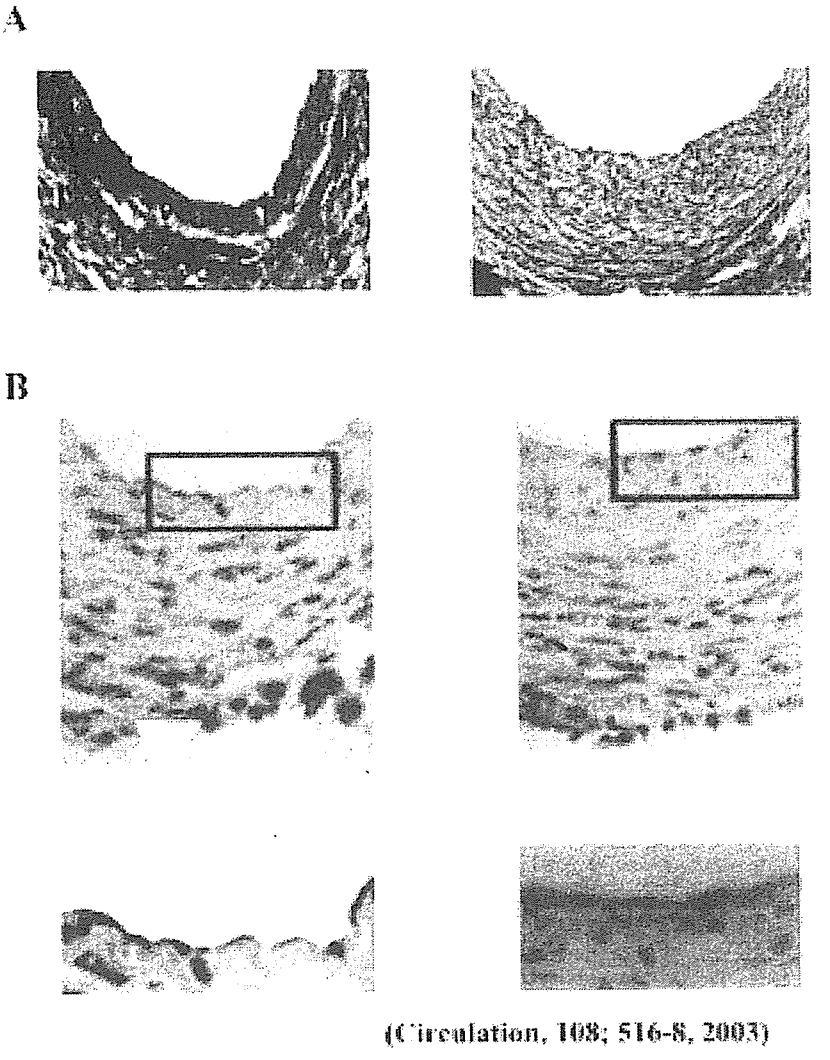
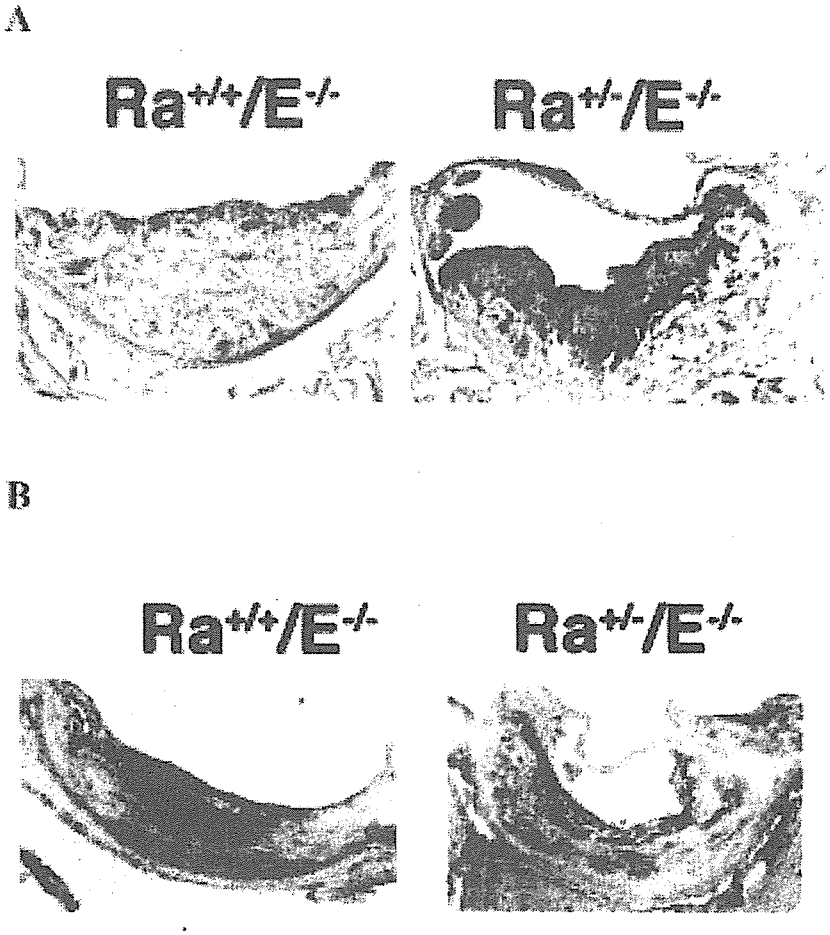


Fig.1. Lack of IL-1Ra promotes neointimal formation after injury
A. Histology of cuffed femoral arteries (day 21) of IL-1Ra$^{+/+}$ (left) and IL-1Ra$^{-/-}$ (right) mice. Intimal thickness was significantly increased in IL-1Ra$^{-/-}$ mice compared with IL-1Ra$^{+/+}$ mice. Sections were stained with Masson's trichrome. Original magnification X 50.
B. Representative photomicrographs depict immunohistochemical staining for IL-1Ra in cuffed femoral arteries of IL-1Ra$^{+/+}$ (left) and IL-1Ra$^{-/-}$ (right) mice (day 7). Boxed areas are enlarged at the bottom of the panels. IL-1Ra protein is present in both the endothelium and some inflammatory cells (arrows) in IL-1Ra$^{+/+}$ mice. Original magnification X 100 (upper panels) and X150 (bottom panels).

counter-regulated by its endogenous inhibitor IL-1Ra (15, 27) and a previous report showed that IL-1Ra is expressed in ECs and atherosclerotic lesions (18). Treatment with recombinant IL-1Ra proved an effective therapy for atherosclerosis in apoE$^{-/-}$ mice (35). Furthermore, low density lipoprotein receptor-deficient (LDLR$^{-/-}$) mice crossed with transgenic mice expressing high levels of murine sIL-1Ra were also partially protected compared to their non-transgenic controls. In contrast, LDLR$^{-/-}$IL-1Ra$^{-/-}$ mice had a tendency to develop foam cell lesions on a diet rich in cholesterol and cholate (36). Moreover, in humans IL-1Ra gene polymorphism is significantly associated with coronary artery disease (37). These findings suggest that endogenous IL-1Ra may suppress atherosclerosis. To directly answer the question of whether a deficiency of IL-1Ra promotes the development of atherosclerotic lesions and/or can modulate the phenotype of atheroma, we employed IL-Ra$^{-/-}$ mice. Using apoE$^{-/-}$ mice as an animal model of atherosclerosis, we established three genotypes (IL-1Ra$^{+/+}$/ apoE$^{-/-}$, IL-1Ra$^{+/-}$/ apoE$^{-/-}$, and IL-1Ra$^{-/-}$/ apoE$^{-/-}$ mice) by cross-breeding. This study focused on the comparison of atherosclerotic lesions and IL-1Ra$^{+/+}$/ apoE$^{-/-}$ and IL-1Ra$^{+/-}$/ apoE$^{-/-}$ mice, because of the significantly leaner phenotype in IL-1Ra$^{-/-}$/ apoE$^{-/-}$ mice. Interestingly, the size of the atherosclerotic lesion safter 16 weeks was significantly increased (30%) in IL-1Ra$^{+/-}$/ apoE$^{-/-}$ mice compared to IL-1Ra$^{+/+}$/ apoE$^{-/-}$ mice (38). Following 32 weeks, the differences in lesion size between these mice failed to achieve statistical significance (38). However, immunostaining demonstrated an 86% increase in the MOMA-2-stained area in IL-1Ra$^{+/-}$/ apoE$^{-/-}$ mice (Fig. 2A). In addition, α-actin stain-



(Arterioscler Thromb Vasc Biol. 24: 1068-73, 2004)

Fig.2. Deficiency of IL-1Ra modulates plaque composition in ApoE$^{-/-}$ mice.
A. Representative photomicrographs of sections of advanced atherosclerotic plaques (immunohistochemical staining for MOMA-2) from the aortic sinus of IL-1Ra$^{+/+}$/apoE$^{-/-}$ (Ra$^{+/+}$/E$^{-/-}$) (left) and IL-1Ra$^{+/-}$/apoE$^{-/-}$ (Ra$^{+/-}$/E$^{-/-}$) (right) mice at 32 weeks of age. IL-1Ra$^{+/-}$/apoE$^{-/-}$ mice showed markedly increased numbers of macrophages in lesions compared with IL-1Ra$^{+/+}$/apoE$^{-/-}$ mice. Original magnification X100.
B. Representative photomicrographs of sections of advanced atherosclerotic plaques (immunohistochemical staining for α-smooth muscle cell actin) from the aortic sinus of IL-1Ra$^{+/+}$/apoE$^{-/-}$ (Ra$^{+/+}$/E$^{-/-}$) (left) and IL-1Ra$^{+/-}$/apoE$^{-/-}$ (Ra$^{+/-}$/E$^{-/-}$) mice (right) at 32 weeks of age. SMC numbers in the lesions in IL-1Ra$^{+/-}$/apoE$^{-/-}$ mice were significantly decreased compared with those in IL-1Ra$^{+/+}$/apoE$^{-/-}$ mice. Original magnification X100.

ing in these lesions was significantly decreased (–15%) compared to that in IL-1Ra$^{+/+}$/ apoE$^{-/-}$ mice (Fig. 2B) (38). Our real-time polymerase chain reaction (RT-PCR) analysis revealed that deletion of IL-1Ra increases the mRNA expression of the adhesion molecules vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 in the aorta, and enhances mRNA levels of monocyte chemoattractant protein (MCP)-1 (38). These changes may contribute to the enhanced accumulation of Mφs in advanced plaques. Interestingly, IL-1β$^{-/-}$ / apoE$^{-/-}$ mice showed opposite results, thus demonstrating that the size of atherosclerotic lesions at the aortic sinus in IL-1β$^{-/-}$ /apoE$^{-/-}$ mice at 12 and 24 weeks of age showed a significant decrease of 30% compared with that in IL-1β$^{+/+}$ /apoE$^{-/-}$mice, and the mRNA levels of VCAM-1 and MCP-1 in the IL-1β$^{-/-}$ /apoE$^{-/-}$ aorta were significantly reduced compared with those in the IL-1β$^{+/+}$ /apoE$^{-/-}$ mice (39). They suggested that IL-1β exerts an atherogenic action by enhancing the expression of VCAM-1 and MCP-1 in the aorta. This report may support our results. Taken together, these findings suggest an important role for IL-1Ra in suppressing the development of lesions early during atherogenesis and furthermore, implicate it in the modulation of plaque composition.

IL-1Ra and cholesterol metabolism during chronic inflammation

Infection and inflammation induce an acute-phase response (APR) (40), leading to multiple alterations in lipid and lipoprotein metabolism (41). Serum triglyceride (TG) levels are increased by multiple cytokines, including IL-1, IL-2, IL-6 and tumor necrosis factor (42–48), because of increased secretin of very low density lipoprotein

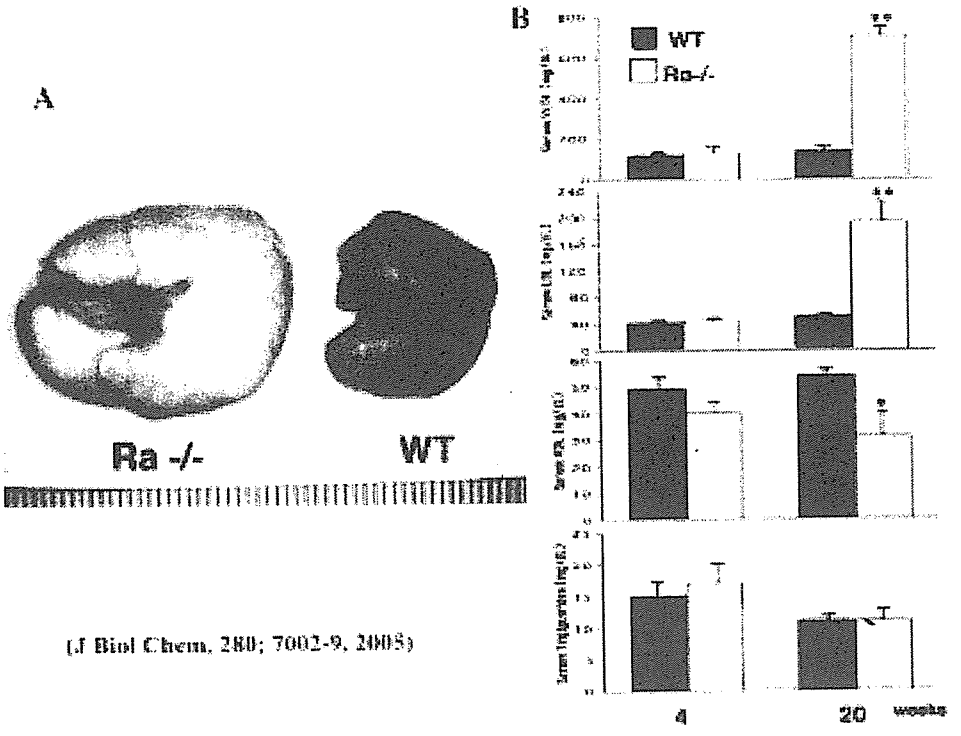


Fig.3. IL-1Ra $^{-/-}$ mice showed severe fatty liver and hypercholesterolemia following 20 weeks on an atherogenic diet when compared with IL-1Ra$^{+/+}$ mice.
A. Macroscopic appearance of livers from IL-1Ra$^{-/-}$ (Ra$^{-/-}$) and IL-1Ra$^{+/+}$ (WT) mice fed an atherogenic diet for 20 weeks. There was a prominent change in color and size in the liver of IL-1Ra$^{-/-}$ versus IL-1Ra$^{+/+}$ mice.
B. Plasma levels of VLDL (upper), LDL (middle upper), HDL (middle lower) and triglycerides (lower) in IL-1Ra$^{-/-}$ (Ra$^{-/-}$) and wild-type (WT) mice. All values are expressed as the mean + SEM. * $p <$ 0.05, ** $p < 0.01$ for Ra$^{-/-}$ mice versus WT mice.

(VLDL) as a result of adipose tissue lipolysis (49–51). With more severe inflammation, the clearance of VLDL decreases secondary to decreased lipoprotein lipase and apolipoprotein E in VLDL (52–54). LPS and cytokines reduce total serum cholesterol levels in primates, whereas in rodents they increase cholesterol levels by stimulating de novo cholesterol synthesis (55, 56), decreasing lipoprotein clearance (57), limiting the conversion of cholesterol to bile acids (58, 59), and decreasing the secretion of cholesterol into the bile (60–63). Many of the changes in lipoproteins during inflammation help to protect the host from harmful effects of the stimuli. However, if prolonged, these changes in the structure and function of lipoproteins will contribute to atherogenesis. Of note, inflammatory cytokines are increased and play a pathogenic role in a variety of very common disorders, such as diabetes, obesity, metabolic syndrome, and atherosclerosis (64–68). Many of these disorders display abnormalities in lipid metabolism that are similar to those that occur during infection and inflammation. However, the effect of chronic inflammation on lipid metabolism has been unclear. Furthermore, there is no report that shows the role of IL-1Ra in the metabolism of cholesterol under chronic inflammatory conditions.

To elucidate the role of IL-1Ra, we fed an atherogenic diet (with cholate) to both IL-1Ra$^{-/-}$ and IL-1Ra$^{+/+}$ mice. IL-1Ra$^{-/-}$ mice developed severe fatty liver after 20 weeks compared to IL-1Ra$^{+/+}$ mice (Fig. 3A) (69). Histological examination revealed an increase in the number and size of intracellular vacuoles, portal fibrosis, and collagen deposition as well as lobular and portal inflammation in livers of IL-1Ra$^{-/-}$ mice. Expectedly, the plasma lipid profile became more proatherogenic with increased total cholesterol levels (942 ± 160 mg/dl versus 240 ± 13 mg/