Interleukin-10 Blocks Atherosclerotic Events
In Vitro and In Vivo

Laura J. Pinderski Oslund, Catherine C. Hedrick, Tristana Olvera, Amy Hagenbaugh, Mary Territo,
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Abstract—Atherosclerosis can be viewed in part as an inflammatory disease process and may therefore be susceptible to manipulation of the immune state. Interleukin 10 (IL-10) is an inhibitory cytokine produced by activated lymphocytes and monocytes. These studies present evidence that IL-10 can inhibit minimally oxidized LDL (MM-LDL)–induced monocyte-endothelium interaction as well as inhibit atherosclerotic lesion formation in mice fed an atherosclerotic diet. Pretreatment of human aortic endothelial cells (HAECs) for 18, but not 4, hours with recombinant IL-10 caused a significant decrease in MM-LDL–induced monocyte binding. IL-10 was found to be maximally effective at 10 ng/mL. Transfection of HAECs with adenovirus expressing viral bcrf-1 IL-10 (Ad-vIL-10) in a sense but not antisense orientation completely inhibited the ability of MM-LDL to induce monocyte binding. Similar results were obtained with IL-10 or Ad-vIL-10 in HAECs stimulated with oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC). We have previously shown increases in cAMP associated with MM-LDL activation of endothelial cells. The MM-LDL–induced increase in cAMP levels was not inhibited by preincubation with IL-10. In vivo studies demonstrated that mice with a murine IL-10 transgene under the control of the human IL-2 promoter have decreased lesions versus controls on an atherogenic diet (5433 ± 6 4008 mm² versus 13 574 ± 4212 mm²; P < 0.05), whereas IL-10 null mice have increased lesions (33 250 ± 9117 mm²; P < 0.0001) compared with either controls or IL-10 transgenic mice. These studies suggest an important role for IL-10 in the atherosclerotic disease process. (Arterioscler Thromb Vasc Biol. 1999;19:2847-2853.)

Key Words: atherosclerosis ■ interleukin-10 ■ MM-LDL ■ monocytes

The pathophysiology of atherosclerosis can be approached on the cellular level as a series of inflammatory stimuli and tissue responses.1,2 Although several components of the immune system have been implicated, monocytes stand out as playing a particularly relevant role. Monocytes have been shown to interact with the vessel wall very early in atherogenesis at sites characteristic of subsequent atherosclerotic lesion development.3 Their presence within atherosclerotic lesions leads to elaboration of chemokines, which may attract smooth muscle cells to invade the developing lesion and attract additional monocytes.1,2 Monocytes engulf oxidized lipids within the lesion, elaborating into “foam cells.” Later in lesion development, release of macrophage-derived substances such as metalloproteinases versus tissue inhibitors of metalloproteinases may partially regulate the morbid events associated with plaque instability and rupture.4–7

Oxidized lipoproteins, in particular minimally modified LDL (MM-LDL), have been demonstrated to induce an inflammatory state in endothelial cells.8 MM-LDL and oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (OxPAPC) can lead to the development of a proinflammatory, activated state that may contribute to the development of recognition, adhesion, and migration of immune cells into areas of vascular tissue, leading to atherosclerotic disease.9 In tissue culture, it has been shown that MM-LDL can activate human aortic endothelial cells (HAECs), leading to monocyte adherence. This increased adherence is due to expression of CS-1 containing fibronectin on the apical surface of HAECs.10 MM-LDL has been documented to cause increased production of monocyte chemotactic protein-1 and macrophage colony–stimulating factor, which can lead to monocyte recruitment, proliferation, and differentiation.9,11,12 Furthermore, MM-LDL can increase levels of intracellular cAMP in rabbit aortic endothelial cells and HAECs. H89, an inhibitor of protein kinase A activity, was shown to inhibit monocyte binding to MM-LDL–treated rabbit aortic endothelial cells.13 The biological activity of MM-LDL has been localized to its oxidized phospholipid components.8 One of the lipids contained within MM-LDL, which appears to account for much of its biological activity, is OxPAPC.14 Therefore, some of
our present studies were performed with both MM-LDL and OxPAPC.

In addition to oxidized lipids, a number of cytokines found in atherosclerotic lesions, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon-γ (IFN-γ), may also be regulators of the proinflammatory aspects of atherogenesis. In contrast to these proinflammatory processes, counterregulatory inhibitory mechanisms, such as select cytokines, are present in biological systems. One such inhibitory cytokine is IL-10, which is a pleiotropic cytokine produced by monocytes, lymphocytes, mast cells, and keratinocytes. This cytokine has already been implicated in atherosclerosis: in situ hybridization studies have identified this cytokine within human atherosclerotic lesions, and highly oxidized LDL has been shown to increase production of IL-10 in human monocytes. Our present studies, therefore, were undertaken to determine the effect of the inhibitory cytokine IL-10 on the inflammation that occurs in atherosclerosis. Furthermore, these studies were conducted both in vitro and in murine models of atherosclerosis in vivo to determine the potential modulating impact of IL-10 on this important disease process.

Methods

Cell Preparation

HAECs were isolated and cultured as previously described. Cells from the 3rd through 10th passages, at 90% to 100% confluence, were used for experiments. Peripheral blood mononuclear cells were isolated from blood samples of healthy human volunteers by a modified Recalde method.

Endothelial Adhesion Assay

Monocyte binding to HAECs was carried out as previously described. Briefly, HAECs were cultured until confluent (72 hours) in gelatin-coated 48-well tissue culture plates. They were then stimulated or treated as described in the various experiments. The treatment or stimulatory agents were aspirated, and the HAECs were washed 3 times. Monocytes (contaminated with lymphocytes, mast cells, and keratinocytes), at a concentration of 106/mL in a final volume of 300 μL, were then added to the wells and incubated for 15 minutes at 37°C; humidified with 5% CO2. Nonadherent cells were rinsed off with 2 washes in 0.5 mL EC medium (20% FBS in medium 199; Gibco), and the cells were fixed in 1% glutaraldehyde. The number of adherent cells was then determined by inverted phase-contrast microscopy at ×250 magnification and a 10×10-mm grid. The number of adherent cells was assessed by counting 3 uniformly covered central fields in 3 identically treated wells. We have previously shown that binding of unstimulated lymphocytes to either unstimulated or stimulated HAECs is minimal; thus, lymphocytes contribute negligibly to the bound cells.

Lipoprotein Preparation

Lipoproteins were isolated and prepared as previously described.

Adenoviral Vectors

Recombinant adenovirus containing β-galactosidase (Adβgal) was generously supplied by Robert Meidell, MD, University of Texas Southwestern Medical Center, Dallas. Recombinant adenoviruses containing the Epstein-Barr virus bcr-1 cDNA with IL-10 inserted in the forward (Ad-VL-10) and in the reverse (Ad-RvIL-10) orientations were generously supplied by Arnold Berk, MD, University of California at Los Angeles. Large-scale preparation of adenovirus was performed as previously described.

Infection of HAECs With Recombinant Adenoviruses

Endothelial cells in 48-well plates were infected with Adβgal at multiplicities of infection (MOIs) of 10 to 500 pfu/cell for 72 hours to determine optimum transfection efficiency. For these studies, HAECs infected with Adβgal were fixed at room temperature with 1% glutaraldehyde in PBS for 15 minutes, then rinsed 3 times with PBS. The wells were then incubated with X-Gal (Gibco-BRL) for 30 minutes at 37°C. For experiments in which monocyte binding was tested, IL-10 or β-galactosidase containing adenoviruses were incubated with HAECs at an MOI of 30 in 48-well dishes for 48 hours. Mononuclear cell adhesion assays were performed 24 hours later. In separate wells, medium was removed 48 hours after infection for measurement of IL-10 secretion with an ELISA assay (R&D Systems).

cAMP Measurement

HAECs were cultured in 6-well plates. At ~90% confluence, wells were pretreated with IL-10 (2 U/mL) for 18 hours. Cells were then treated with 1 mmol/L isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor, for 10 minutes. MM-LDL, cholestrol toxin, and IL-10 were then added to the various wells. After treatment, the cells were rinsed with cold 1% PBS containing 4 mmol/L EDTA. PBS/EDTA (0.5 mL) was then added to each well, and cells were collected by scraping. The wells were rinsed with a second aliquot of PBS/EDTA and transferred to the same microfuge tube. The cells were then microcentrifuged at room temperature for 5 minutes. The supernatant was aspirated off, and 200 μL of boiling 50 mmol/L sodium acetate, pH 5.8, was added. Cells were then sonicated for 20 seconds. Each sample was boiled in a water bath for 5 minutes and then centrifuged for 3 minutes at high speed to remove proteins. The supernatant was then transferred to fresh tubes and stored at −8°C until further use. For cAMP assay, 50 μL of the supernatant was measured in duplicate as previously described with a β2L-labeled kit from Amersham.

IL-10 Transgenic and Null Mouse Models

A transgenic mouse model overexpressing murine IL-10 under the control of the human IL-2 promoter was constructed as previously described. These mice were serially backcrossed for >6 generations onto a C57BL/6J background. All mice used were congenic on the C57BL/6J background. Seven wild-type C57BL/6J, 7 IL-10 transgenic, and 9 IL-10 homozygous null mice (Jackson Laboratory, Bar Harbor, Me) were housed under specific pathogen–free conditions and fed an atherogenic diet (15% saturated fat, 1.25% cholesterol, 0.5% cholic acid) for 15 weeks. The animals were then fasted overnight, blood samples were obtained for lipid analysis, and the animals were euthanized. The aortas were immediately removed and frozen in OCT medium over dry ice, then fixed, sectioned, and stained with oil red O. The atherosclerotic lesions were quantified as previously described.

Statistical Analysis

All statistical analysis was performed with Student’s t tests with StatView (Abacus Concepts, Inc). Data are presented as mean±SD.

Results

IL-10 Inhibits Monocyte Adhesion to HAECs

In Vitro

To determine the potential effect of exogenous IL-10 on monocyte adhesion to activated HAECs, a series of in vitro experiments was carried out. Our laboratory has previously published that incubation of HAECs with the oxidized lipids MM-LDL8 or OxPAPC leads to an increase in monocyte adhesion. There is a dose-dependent increase in monocyte adherence with increasing MM-LDL concentration, with maximal effect at 250 μg/mL of MM-LDL; Figure 1A. Incubation of HAECs with IL-10 alone at a concentration from 10 ng/mL for 4 to 18 hours did not
affect monocyte–endothelial cell interactions, which suggests that IL-10 is not cytotoxic at this concentration for these time periods (Figure 1B). HAECS pretreated for 18 hours with IL-10 (10 ng/mL) and then stimulated with MM-LDL (250 μg/mL) showed a significant decrease in monocyte adhesion versus MM-LDL–stimulated cells that received no IL-10. In contrast, pretreatment with IL-10 for 4 hours did not significantly alter the increased monocyte binding that occurred on MM-LDL stimulation (Figure 1B). Dose-response curves for IL-10 stimulation (Figure 1C); therefore, this dose was used for the in vitro experiments. Similar results were obtained with experiments carried out with OxPAPC (125 μg/mL) used as the stimulus in place of MM-LDL (Figure 1D). Importantly, the IL-10 was always removed by washing before introduction of the

Figure 1. IL-10 blocks monocyte adhesion to HAECS stimulated with oxidized lipoproteins. A, Dose-response curve of monocyte binding to HAECS stimulated with increasing amount of MM-LDL. B, IL-10 (10 ng/mL) pretreatment for 18 hours blocks monocyte adhesion to HAECS induced by MM-LDL (250 μg/mL), whereas 4 hours of IL-10 pretreatment is insufficient. C, Dose-response curve demonstrating inhibitory effect of IL-10 on monocyte adhesion to HAECS stimulated with MM-LDL. D, IL-10 (10 ng/mL) pretreatment for 18 hours blocks monocyte adhesion to HAECS induced by OxPAPC (125 μg/mL). Each of these panels is representative of 3 experiments giving similar results. Nine fields were examined for each data point. Data represent mean±SD; *P<0.05.
monocytes; therefore, these data represent an effect on the HAECs and not an effect on the monocytes used for these adhesion assays.

**Effect of Adenovirus-Mediated Overexpression of IL-10 on Oxidized Lipoprotein–Induced Monocyte Binding**

To further examine the effect of IL-10 on endothelial cell activation caused by oxidized lipoproteins, adenoviral experiments were undertaken to overexpress IL-10 in HAECs. We used an adenovirus overexpressing bcrf-1 viral IL-10 (Ad-vIL-10), which has previously been shown to have fewer immunostimulatory effects than human IL-10. HAECs were transfected at an MOI of 30 with Ad-vIL-10 for 72 hours before stimulation with oxidized lipoproteins. The level of IL-10 overexpression achieved by Ad-vIL-10 at an MOI of 30 was confirmed by ELISA and was found to be ≈4 ng/mL in the cell culture supernatant. Levels of IL-10 were ∼0 ng/mL in the cell culture supernatant without transfection or with control adenovirus expressing β-galactosidase. Transfection of HAECs with Ad-vIL-10 inhibited MM-LDL–induced monocyte-endothelium interaction (Figure 2). Similarly, adenoviral overexpression of IL-10 blocked OxPAPC–induced monocyte binding (data not shown). In both cases, a control adenovirus overexpressing β-galactosidase did not block the lipoprotein-induced monocyte adhesion. Thus, we conclude that the delivery of viral bcrf-1 IL-10 via this vector is comparable to exogenous delivery of recombinant human IL-10, and these data indicate that IL-10 expression blocks MM-LDL– and OxPAPC–induced monocyte adhesion to HAECs.

**IL-10 Does Not Alter cAMP Levels in HAECs**

MM-LDL–has previously been shown to increase cAMP levels in HAECs. To determine whether IL-10 exerts its inhibitory effects by blocking this rise in cAMP, studies were conducted with MM-LDL–stimulating HAECs, and cAMP levels were measured. IL-10 by itself does not increase cAMP, nor does it block the MM-LDL–induced increase in cAMP under identical conditions in which it inhibits the monocyte binding described above (Figure 3). Therefore, it appears that IL-10 acts at some point downstream of the rise in cAMP.

**IL-10 Overexpression Reduces Atherosclerotic Lesion Development in Mice**

Studies were conducted using murine models to investigate the impact of IL-10 on the development of atherosclerotic lesions in vivo. Three groups of mice were used: wild-type C57BL/6J controls (n = 7), IL-10 transgenic mice (n = 7), and IL-10 homozygous null mice (n = 9). The IL-10 transgenic mice express murine IL-10 under the control of the human IL-2 promoter. T lymphocytes from these IL-10 transgenic mice overexpress IL-10 2-fold to 4-fold, resulting in a shift of the T lymphocyte population to a TH2 cytokine–producing phenotype, with decreased IFN-γ production. All mice were on a C57BL/6J background. After 15 weeks on the atherogenic diet, the total cholesterol was not significantly different among wild-type, IL-10 transgenic, and IL-10 null mice. The HDL cholesterol was comparable between the wild-type and transgenic groups; however, it was significantly lower in the IL-10 null group (Table). There was a marked difference in lesion size among the 3 groups, with the IL-10 transgenic mice displaying significantly less atherosclerotic lesion formation than either wild-type or IL-10 null mice. The lesions in the wild-type and IL-10–deficient animals were fatty-streak lesions with accumulation of small mononuclear cells and lipid; no fibrous caps were observed at the time of the study (Figure 4). Aortic lesion formation was reduced in the IL-10 transgenic mice to a mean of 5433 ± 4008 mm² versus 2850 Arterioscler Thromb Vasc Biol. December 1999

**Figure 2.** Effect of an adenovirus overexpressing IL-10 (AdIL-10) and recombinant human IL-10 (IL-10) on monocyte adhesion to MM-LDL–stimulated HAECs. Adenovirus overexpressing β-galactosidase (β-gal) and AdIL-10 (at MOI 30) were incubated for 48 hours in dishes confluent with HAECs. Monocyte binding assays, with MM-LDL (250 μg/mL) stimulation for 3 hours, were performed 24 hours after completion of virus incubation. IL-10 (10 ng/mL) also significantly blocked monocyte-HAEC adhesion under identical stimulatory conditions. These data are representative of 3 experiments that gave similar results. Nine fields per well were counted. Data represent mean ± SD; *P < 0.05.

**Figure 3.** IL-10 does not block the increase in cAMP in HAECs caused by stimulation with MM-LDL. HAECs were pretreated with IL-10 (10 ng/mL) for 24 hours, then treated with 1 mmol/L IBMX for 10 minutes, with subsequent stimulation with MM-LDL (250 μg/mL) for 3 hours. Cells were then collected and cAMP measurements performed. Data are presented as percent increase over control, with control levels at 200 fmol/well. Data are average of 4 experiments in which each condition was tested in triplicate. Data represent mean ± SD; *P < 0.05.

<table>
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<tr>
<th>Plasma Lipoproteins in Various Mouse Groups</th>
<th>Cholesterol</th>
<th>HDL</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>323 ± 76</td>
<td>67 ± 15</td>
</tr>
<tr>
<td>IL-10 transgenic</td>
<td>312 ± 80</td>
<td>62 ± 10</td>
</tr>
<tr>
<td>IL-10 knockout</td>
<td>381 ± 76</td>
<td>40 ± 16*</td>
</tr>
</tbody>
</table>

Plasma lipoproteins measured at time of death. Values are in mg/dL.

*P < 0.05.
Figure 4. IL-10 transgenic mice have significantly less fatty-streak development. Aortic root sections taken from C57BL/6J (A and B), IL-10 transgenic (C and D), and IL-10–deficient (E and F) mice stained with oil red O. Left, magnification ×10; right, magnification ×40.

IL-10 levels affect atherosclerotic lesion formation in vivo. C57BL/6J wild-type (wt; n = 7), IL-10 transgenic (Tg; n = 7), and IL-10 null (KO; n = 9) mice were fed an atherogenic diet for 15 weeks. Aortic lesions were significantly reduced in the IL-10 Tg mice compared with the wt controls (5433 ± 4008 vs 13 574 ± 4212 mm²; \( P < 0.05 \); Figure 5). Conversely, the mice homozygous null for IL-10 had a marked increase in lesion area (33 250 ± 9117 mm²; \( P < 0.0001 \); Figure 5).

Comparison of lesion size between IL-10 transgenic and IL-10 null mice revealed a marked difference in the atherosclerotic disease state (5433 ± 4008 vs 33 250 ± 9117 mm²; \( P < 0.0001 \)). Thus, there appears to be a significant and inversely related effect of IL-10 on the atherosclerotic process in vivo.

Figure 5. IL-10 levels affect atherosclerotic lesion formation in vivo. C57BL/6J wild-type (wt; n = 7), IL-10 transgenic (Tg; n = 7), and IL-10 null (KO; n = 9) mice were fed an atherogenic diet for 15 weeks. Aortic lesions were significantly reduced in the IL-10 Tg mice compared with the wt controls (5433 ± 4008 vs 13 574 ± 4212 mm²; \( P < 0.05 \)). IL-10 KO mice had a marked increase in lesion area versus wt or Tg animals (33 250 ± 9117 mm²; \( P < 0.0001 \)).

Discussion

IL-10 has been shown to inhibit a broad range of immunological mediating processes.\(^{32}\) The action of this pleiotropic cytokine has been found to be cell-type–specific,\(^{33}\) with activating effects on CD8\(^+\) T cells\(^{37}\) contrasting with inhibiting effects on monocytes\(^{34}\) and some T-cell subsets.\(^{22}\) In our experimental system, we have found that IL-10 can inhibit the stimulatory effects of the oxidized lipids MM-LDL and OxPAPC (Figures 1A and 1B) as well as those of cholera toxin (data not shown) on HAECs, manifested by an inhibition of monocyte adhesion after 18 hours of IL-10 pretreatment. We have also demonstrated that recombinant human IL-10 and viral bcrf-1 IL-10 are both able to achieve this effect. The lengthy pretreatment necessary in our studies for the inhibitory effect contrasts markedly with the ability of IL-10 to immediately inhibit lipopolysaccharide or TNF-α activation of monocytes.\(^{34}\) Because of the length of endothelial pretreatment required, it is likely that transcriptional or translational processes are involved. Previous work from our laboratory has shown that MM-LDL exerts its effects by initiating an increase in intracellular cAMP.\(^{35}\) We therefore examined cAMP levels in the presence of IL-10 after stimulation with MM-LDL (Figure 3) or cholera toxin (data not shown) and found them to be elevated to levels equivalent to those in cells identically stimulated but not treated with IL-10. This study suggests that IL-10 acts downstream of the MM-LDL–mediated increase in cAMP.

IL-10 has been documented to play an important role in inflammatory disease processes in mice. IL-10–deficient mice spontaneously develop a severe form of enterocolitis,\(^{16,36}\) Adoptive transfer of CD45RB\(^+\) CD4\(^+\) T cells from IL-10 transgenic mice, however, is able to suppress inflammatory colitis development in SCID mice under conditions that would normally cause this form of intestinal inflammation.\(^{17}\) Recombinant inbred BALB/c mice challenged with the intracellular pathogen Chlamydia produce higher levels of IL-10 than do C57BL/6J mice. BALB/c mice subsequently mount a less aggressive inflammatory response and consequently succumb to this infection in greater numbers than an identically treated C57BL/6J group.\(^{37}\) IL-10–deficient mice infected with Chlamydia in sublethal doses develop less granulomatous formation than wild-type mice.\(^{38}\)

On the basis of these studies in mice and our results in HAECs in culture, we next examined the effect of IL-10 on atherosclerosis in mice. In both IL-10 transgenic and null mutation mouse models, we investigated the effect of overproduction compared with normal or absent production of this inhibitory cytokine on the development of atherosclerotic lesions with the mice on an atherogenic diet. We found that IL-10 was able to significantly reduce the development of atherosclerosis in the mice studied (Figures 4 and 5) without affecting total plasma cholesterol. Although HDL cholesterol was not different between wild-type and transgenic mice, we cannot exclude the possible role the reduced HDL levels might have played in the IL-10 null mice (Table). IL-10 null mice had the largest amount of lesion burden, which decreased in wild-type mice and decreased further in mice transgenic for IL-10. Although we did not measure the levels of IL-10 in our mice, previous studies have shown that IL-10 is not detectably increased in the circulating plasma of the...
IL-10 transgenic mice at 4 weeks of age.\textsuperscript{17} This study suggests that in the transgenic mice in which IL-10 is under control of the human IL-2 promoter, IL-10 is increased at the level of the individual lymphocytes. This decrease in the fatty-streak formation in the IL-10 transgenic group may be important because of the still unresolved controversy concerning the role of lymphocytes in atherosclerosis. Several conflicting studies argue for or against the relevance of T lymphocytes in the development of atherosclerosis. For instance, the finding that SCID and RAG-2–deficient mice are still able to achieve significant levels of atherosclerosis despite not having lymphocytes\textsuperscript{39–41} appears to be in contrast to other studies that demonstrate early infiltration and activation of lymphocytes within atherosclerotic lesions.\textsuperscript{5,7,42–44} Other studies demonstrate a key role for TH1 cytokines, such as IL-12\textsuperscript{45} or IFN-\gamma,\textsuperscript{46} in atherogenesis. A compelling study performed in apolipoprotein E–deficient mice revealed a striking immunological shift from a predominantly TH1 to a TH2 phenotype with the stress of high-cholesterol feeding, as evidenced by immunoglobulin subclasses and cytokine production.\textsuperscript{47}

Our data with transgenic mice are consistent with a role for lymphocytes in atherogenesis. Whether the decreased atherosclerotic lesion formation in our studies is a direct effect of the lymphocytes themselves or a secondary effect of the lymphocyte IL-10 or the lymphocyte subpopulation on monocytes, endothelial cells, or other cells is not addressed in our in vivo studies. Our data nevertheless strongly support the role of IL-10 in atherosclerosis. Further work focused on this cytokine may reveal important mechanisms in the pathogenesis of atherosclerosis.

Note Added In Proof

After this manuscript was accepted for publication, a manuscript appeared displaying similar findings for IL-10–deficient mice fed an atherogenic diet. (Mallet et al, \textit{Circ Res}. 1999;85:e17–e24).

Acknowledgments

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