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Expanding expression of the 5-lipoxygenase/leukotriene B_4 pathway in atherosclerotic lesions of diabetic patients promotes plaque instability

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Abstract

Emerging evidence now indicates that the 5-lipoxygenase (5-LO) pathway play a role in the pathogenesis of atherosclerosis and restenosis. The expression of 5-LO by activated macrophages in symptomatic plaques leads to leukotriene B_4 (LTB_4) accumulation and enhanced synthesis and release of matrix metalloproteinases (MMPs) that can promote plaque rupture. However, the role of 5-LO pathway in diabetic vascular disease has not been previously reported. Thus, the present study was designed to analyze the expression of 5-LO in carotid plaques of diabetic patients and to investigate the possible role of 5-LO pathway in the pathogenesis and progression of diabetic atherosclerosis. Atherosclerotic plaques from 60 patients undergoing carotid endarterectomy were divided into non-diabetic and diabetic group. Plaques were analyzed for 5-LO, MMP-2 and MMP-9 by immunohistochemical, Western blot, and densitometric analyses, whereas zymography was used to detect MMP activity. Immunocytochemistry was also used to identify CD68+macrophages, CD3+T-lymphocytes, and HLA-DR+inflammatory cells. LTB_4 were quantified by enzyme-linked immunosorbent assay. 5-LO showed abundant immunoreactivity in human atherosclerotic carotid lesions, and was colocalized with macrophage infiltrates in atherosclerotic intima. 5-LO expression was higher in diabetic compared with non-diabetic plaques and was associated with increased MMP-2 and MMP-9 expression. Follow-up analyze with zymography assay revealed MMP activity was elevated in diabetic compared with non-diabetic plaques. Notably, in contrast to non-diabetic plaques, LTB_4 levels were significantly increased in diabetic plaques by enzyme-linked immunosorbent assay. These results suggest that overexpression of 5-LO and LTB_4 in atherosclerotic plaques possibly promote MMP-induced plaque rupture in diabetes. Hence, anti-LTs may be useful, not only in reducing atherogenesis, but also in the prevention and treatment of acute atherothrombotic events in diabetic patients.

Keywords: Lipoxygenase; Leukotrienes; Plaque; Diabetes mellitus; Matrix metalloproteinases

Diabetes is associated with significantly accelerated rates of cardiovascular complications such as atherosclerosis. There is increasing evidence that atherosclerosis is an inflammatory disease and that certain inflammatory markers may be key predictors of diabetic atherosclerosis. Inflammatory processes play a pivotal role in the pathogenesis of atherosclerosis, particularly in the progression of atherosclerotic plaque toward instability [1]. More recently; accumulating evidence indicates that inflammatory responses can also be elicited by smaller oxidized lipids that are components of atherogenic oxidized low-density lipoprotein or products of phospholipase activation and arachidonic acid metabolism. These include oxidized lipids of the lipoxygenase and cyclooxygenase pathways of arachidonic acid and linoleic acid metabolism [2]. 5-lipoxygenase (5-LO) metabolizes free arachidonic acid leading to the formation of proinflammatory leukotrienes (LTs). The participation of 5-LO in the development of atherosclerosis has been suggested by recent studies. Furthermore, a recent

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study reported that the participation of 5-LO in atherosclerosis occurs not only during the development of atherosclerotic plaques but also during the progression of atherosclerotic plaques toward instability [3]. 5-LO is a marker for increased risk of acute ischemic events precipitated by MMP-dependent processes that lead to plaque rupture.

LTs are a group of proinflammatory lipid mediators that are implicated in the pathogenesis and progression of atherosclerosis. A growing body of morphological, biochemical, pharmacological, and functional evidence collected from human and animal studies has implicated LTs in the development and progression of atherosclerosis, in particular LTB_4 [4–7]. LTB_4 is one of the downstream products of the 5-LO pathway and it is known to function as a potent chemoattractant and proinflammatory mediator in the pathogenesis of several inflammatory diseases including atherosclerosis [8–10]. LTB_4 may be one of the key mediators of 5-LO-dependent plaque instability. The overexpression of 5-LO by activated macrophages in symptomatic plaques leads to LTB_4 accumulation and enhanced biosynthesis and release of MMPs that can promote plaque rupture [3]. Although recent studies have demonstrated an important role for 5-LO pathway in atherosclerosis, little is known about the effects of 5-LO pathway in diabetic vascular disease.

Because the prevalence of cardiovascular disease is significantly greater in diabetic patients than in non-diabetic subjects, we hypothesized that 5-LO pathway may be preferentially activated in the setting of diabetes. In the present study, we examined expression of 5-LO, MMP-2, and MMP-9 in human carotid plaque from diabetic patients. In addition, we compared the LTB_4 levels in diabetic plaques with those in non-diabetic plaques. Our findings indicate the expression of 5-LO pathway is preferentially enhanced in diabetic plaques, atherosclerotic plaques are prone to disrupt and trigger acute atherothrombotic events during hyperglycemia.

Materials and methods

Recruitment of patients. Human atherosclerotic plaques were obtained from 30 non-diabetic patients and 30 diabetic patients undergoing carotid endarterectomy for extracranial high-grade (>70%) internal carotid artery stenosis (Table 1). The degree of luminal narrowing was determined by repeated Doppler echography and intra-arterial cerebral angiography using the criteria of the North American Symptomatic Carotid Endarterectomy Trial [11]. Clinical evidence of plaque instability was provided by the assessment of recent ischemic symptoms attributable to the stenosis and by the presence of ipsilateral cerebral lesion(s) determined by computed tomography. The diabetic group consisted of 26 patients with symptomatic carotid artery disease such as transient ischemic attack (TIA) or stroke. The non-diabetic group included 14 patients who had clinical symptoms of cerebral ischemic attack. The median age of the non-diabetic patients was 50.7 ± 8.6 years old and ranged from 41 to 65 years. The mean age of the diabetic patients was 51.5 ± 9.8 years (range 38–67 years). HbA_{1c} concentrations were higher in the diabetic group ($7.8 \pm 0.2\%$) than in the non-diabetic patients ($5.1 \pm 0.4\%$). The mean duration of diabetes mellitus was 5.2 ± 3.7 years. Written informed consent was obtained from all subjects, and the investigation was approved by the Ethical Committee of China Medical University and was in agreement with institutional guidelines.

Table 1
Characteristics of study patients

Variable	Diabetic ($n = 30$)	Non-diabetic ($n = 30$)
Age, y	51.5 ± 9.8	50.7 ± 8.6
Sex, M/F	16/14	17/13
BMI (kg/m^2)	25 ± 4	24 ± 4
Patients with, *n*		
Recent TIA and stroke	26	14
Family history of IHD	20	18
Hypertension	22	21
Duration of hypertension (y)	5.3 ± 3.2	4.8 ± 3.7
HbA_{1C} (%)	7.8 ± 0.2	5.1 ± 0.4
Cigarette smoking	15	16
Hypercholesterolemia	19	17
NSAID or glucocorticoid treatment	0	0
Treatment with ACE inhibitors or AT_1 receptor blockers	21	20
Aspirin	7	5
Statin	12	9
Glitazone	3	0
Metformin	5	0
Insulin	4	0
Stenosis severity (%)		
Mean ± SD	76 ± 5	77 ± 7
Range	70–91	70–94
Plaque ulceration, *n* (%)	24(80)*	17(57)
Intraplaque hemorrhage, *n* (%)	14 (47)	14 (47)
Percentage of macrophage-rich areas	23 ± 9†	17 ± 6
Number of T cells per mm^2 section area	68 ± 27†	46 ± 19

TIA, transient ischemic attack; IHD, ischemic heart disease; NSAID, non-steroidal anti-inflammatory drug; ACE, angiotensin converting enzyme; AT_1, angiotensin type 1.
* $P < 0.05$.
† $P < 0.01$.

Samples and preparation. Samples of atherosclerotic tissue collected from patients undergoing carotid endarterectomy were washed in PBS and divided longitudinally into two pieces. One was mounted in OCT medium for cryosectioning, snap-frozen on dry ice, and stored at −80 °C until immunohistochemical analysis., and the other was immediately frozen and stored at −80 °C until required for the *in vitro* analysis.

Immunohistochemical staining. The anti-5-LO polyclonal antibody and monoclonal antibodies against human MMP-2 and MMP-9 (Santa-Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used as the primary antibodies for immunochemistry. Peroxidase-conjugated secondary antibodies were used with these primary antibodies. To characterize the type of cells, anti-CD68, anti-CD3, and anti-HLA-DR were used for the immunostaining of macrophages, T-lymphocytes, inflammatory cells, respectively. The paraffin sections were deparaffinized and rehydrated and then boiled with citric acid for 5 min in order to reduce the non-specific binding of antibody and to increase the exposure of antigens, and finally cooled at room temperature for 20 min. Subsequently, the sections were treated with 0.3% H_2O_2 for 5 min to suppress endogenous peroxidase activity. After treatment with PBS (pH 7.2–7.4) for 5 min and application of 1:100 diluted 5-LO, MMP-2, and MMP-9 primary antibodies, the sections were incubated in a moist chamber for 1 h. After washing and bathing for 5 min by PBS, the biotinylated secondary antisera cocktail including goat anti-mouse and anti-rabbit IgG diluted 1:400 was incubated on the slides for 15 min at room temperature in a moist chamber. The sections were then processed by the streptavidin–biotin–peroxidase complex method by use of the LSAB (+) kit (DAKO Inc. Carpenteria, CA, USA) and DAB solution (Research Genetics Inc., Huntsville, AL, USA). The sections were then counterstained with Mayer's hematoxylin.

Quantitative Analysis was performed with a computer-based image analysis system (LUZEX-F, Japan).

Western blot analysis. Tissue extracts (50 μg of total protein/lane) were loaded with Tris-base 0.5 mol/L, 0.8% SDS, 10% glycerol, 0.3% bromophenol blue, and 5% β-mercaptoethanol as sample buffer and electrophoresed on 10% or 14% SDS–polyacrylamide gels. Coimmunodetection of β-actin was performed to ensure equal gel loading. Resolved proteins were transferred onto 0.45-μm nitrocellulose membrane (Amersham Corp.) in a blotting buffer that contained 25 mmol/L Tris–HCl, pH 8.3, 192 mmol/L glycine, 20% methanol, and blocked with 50 mmol/L Tris–HCl, pH 7.5, 150 mmol/L sodium chloride, 0.3% Tween 20 (TBS-T), and 10% non-fat dried milk for at least 2 h. The blots were washed 3 times with TBS-T and then incubated overnight with the primary antibody. After 3 washing steps (3 × 20 min), the blots were incubated for 1 h at 25 °C with a horseradish peroxidase-linked anti-mouse (5-LO, MMP-2, and MMP-9) antibody (Amersham Pharmacia Biotech) at 1:1000 dilution in TBS-T containing 10% non-fat dried milk. The washing steps were repeated, and subsequently, enhanced chemiluminescence detection was performed according to the manufacturer's instructions (ECL Plus, Amersham). Intensities of experimental bands were measured by computer-assisted densitometry.

Gelatin zymograhy. The enzymatic activity of MMP-2 and MMP-9 was measured by gel zymographic analysis. Protein content of the samples was measured by the colorimetric method using serum albumin as the standard. Fifty microgram proteins of atherosclerotic carotid tissue were loaded to 11% SDS–PAGE containing with 0.1% gelatin for electrophoresis under 4 °C cold room. Subsequently, gels were incubated with collagenase buffer for 16 h at 37 °C, stained with 0.25% Coomassie brilliant blue, destained with 30% isopropanol in 10% acetic acid, and visualize. Conditioned medium of human fibrosarcoma cell line HT1080 was used as a positive control with known gelatinolytic activity.

Plaque analysis of LTB_4. Plaque analysis of LTB_4 was performed as previously described [3]. LTB_4 levels were determined in duplicate assays by ELISA (Assay Designs Inc., Ann Arbor, Mich) that had been previously validated in the measurement of LTB_4 in tissue homogenates.

Isolation of macrophages from atherosclerotic plaques. Macrophages were selectively extracted from plaques by enzymatic digestion and density gradient centrifugation according to previously validated methods [12]. The supernatants were then collected and LTB_4 production was determined by ELISA as described above.

Statistics analyses. Data are expressed as percentage or mean ± SD. Clinical and histological variables were compared by χ^2 test. Differences in enzyme expression, enzymatic activity, and inflammatory infiltrate were analyzed by Student's t test. A value of $P < 0.05$ was considered statistically significant. All calculations were performed using the computer program SPSS version 11.5.

Results

Histological analysis

Plaques of both groups had the morphology of advanced lesions, with a lipid-rich acellular core and sites of rupture (71%), superimposed thrombosis, and intraplaque hemorrhage. Plaque ulceration was significantly more common in the diabetic plaques (24 of 30 [80%] versus 17 of 30 [57%]; $P < 0.05$). In contrast, no differences (14 of 30 [47%]) were observed with regard to intraplaque hemorrhage (Table 1).

Inflammatory infiltration

Immunocytochemistry revealed inflammatory infiltration in all specimens examined, more evident in diabetic plaques. Overall, macrophage and T-lymphocyte infiltration occurred coincidentally and was most prominent in the shoulder of the lesions and in the immediate vicinity of the atheromatous core of the lesions. Plaque area occupied by macrophages and T cells was significantly greater ($P < 0.01$) in plaques from the diabetic group than in plaques from the non-diabetic group (Table 1).

Macrophage activation status

The site of inflammatory infiltration in the shoulder of diabetic plaques was always characterized by strong expression of HLA-DR antigens, which contrasted markedly with the low expression of HLA-DR in the non-diabetic plaques. HLA-DR expression was most abundant on macrophages and lymphocytes.

5-LO Is expressed in higher amounts in diabetic plaques

Carotid atherosclerotic lesions showed a thickened intima associated to an area of necrotic core and lipid-laden atheroma in all cases. There was also a prominent infiltration of mononuclear cells and foam cells in the atheromatous plaques. 5-LO was stained within atheromatous plaques, and was colocalized base of atherosclerotic plaque, in the medio-intimal junction area and its immunoreactivity was primarily colocalized with macrophage/mononuclear cells. Carotid plaques displayed abundant expression of 5-LO within the intimal lesion area, with distribution similar to CD68-positive macrophages. Staining for 5-LO was more abundant in diabetic plaques compared with non-diabetic specimens (Fig. 1). Quantitative image analyses revealed that the immunoreactivity of 5-LO in diabetic plaques significantly exceeded those in non-diabetic lesions (29.6 ± 5.2% versus 17.2 ± 3.3%, $n = 30$; $P < 0.05$; Fig. 1).

Western blotting of 5-LO, MMP-2, and MMP-9 were performed in order to define the expression of these proteins quantitatively and to exclude cross reactivity by immunohistochemical staining. Western blot analyses revealed robust expression of 5-LO protein in extracts of diabetic plaque. In diabetic plaque, the expression of the 5-LO was significantly enhanced ($P < 0.05$) compared with non-diabetic atheromatous lesions (Fig. 2).

Higher MMP-2 and MMP-9 expression in diabetic atherosclerotic lesions

MMP-2 and MMP-9 were evident in all cases along with carotid plaques, primarily in macrophages/mononuclear cells and intimal smooth muscle cells. However, the immunohistochemical staining was significantly more abundant in the diabetic compared with the non-diabetic lesions. Quantitative image analyses revealed that the levels of MMP-2 and MMP-9 in diabetic plaques (35.6 ± 4.4% and 37.2 ± 3.6%, respectively; $n = 30$) significantly exceeded ($P < 0.05$) those in non-diabetic plaques

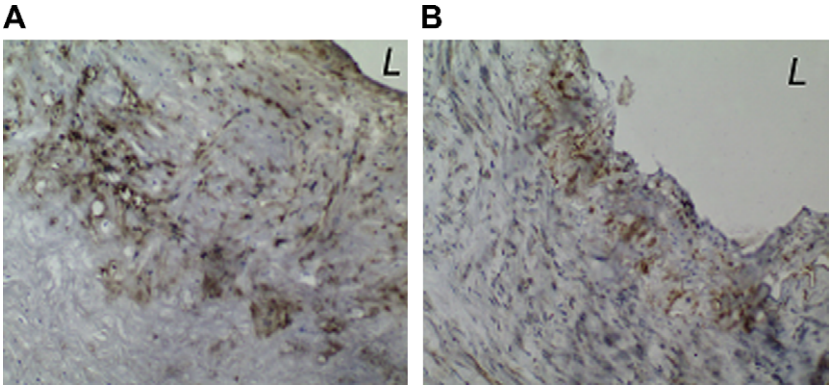

Fig. 1. Expression of 5-LO protein in human atherosclerotic lesions. Immunohistochemical staining revealed more abundant expression of 5-LO protein in diabetic (A) relative to non-diabetic (B) atherosclerotic plaques, colocalizing in macrophages of intimal lesions. These results were typical of 30 non-diabetic and 30 diabetic plaques. *L*, lumen. (Magnification: 400×).

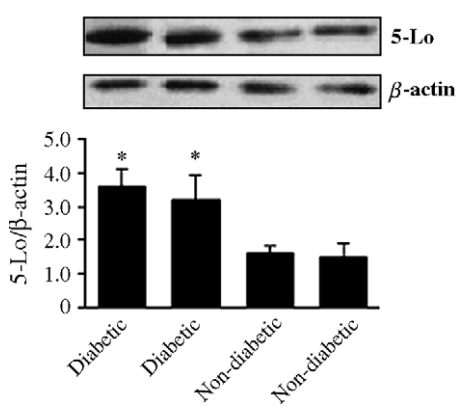

Fig. 2. The expression of 5-LO protein level was measured by Western blot analysis using 5-LO-specific antibodies. The protein bands of 5-LO were indicated. β-Actin expression was used as an internal loading control. The quantitative densitometric scanning results were shown at bottom of panel. 5-LO expression was higher in diabetic compared with non-diabetic atherosclerotic plaques. $^{*}P < 0.05$ as compared with non-diabetic group. The results are representative of studies that included 30 non-diabetic and 30 diabetic plaques.

($25.4 \pm 2.7\%$ and $27.2 \pm 2.3\%$, respectively). Moreover, Increased MMP immunoreactivity was localized around the plaque core, especially in the shoulder and the fibrous cap of the lesions.

Similarly, the increased MMP-2 and MMP-9 immunoreactivity in diabetic versus non-diabetic atherosclerotic lesions was confirmed by Western blot analyses. Western blot analyses revealed MMP-2 and MMP-9 protein levels in diabetic plaque were significantly higher ($P < 0.05$) than those in non-diabetic plaque (Fig. 3A).

Diabetic plaques contain activated MMPs

The robustly increased MMP-2 and MMP-9 immunoreactivity in diabetic versus non-diabetic atherosclerotic lesions was confirmed by Western blot analyses. These changes, however, do not necessarily reflect altered enzymatic activity because MMPs require proteolytic activation for optimal function [13]. Thus, we used SDS–PAGE zymography as a complement to immunohistochemical and immunoblotting studies to assess MMP gelatinolytic activity. Zymographic analysis revealed the amount of inactive and active MMP-2 (4639 ± 273 versus 2734 ± 176 and 2562 ± 232 versus 1738 ± 134 densitometric units [DU], respectively; $n = 30$) and MMP-9 (6242 ± 348 versus 4261 ± 364 and 3845 ± 641 versus 2189 ± 524 DU, respectively) was significantly higher ($P < 0.05$) in the diabetic compared with non-diabetic plaques. Furthermore, Gelatinolytic activity of MMP-9 was more prominent than that of MMP-2 in diabetic atherosclerotic lesions (Fig. 3B).

Cultured macrophages isolated from diabetic and non-diabetic plaques recapitulate differences in 5-LO and MMP levels observed in vivo

To determine whether the higher 5-LO and MMP levels observed in diabetic plaques could account for a higher relative abundance of macrophages in the diabetic specimens. Macrophages were isolated from 8 diabetic and 8 non-diabetic plaques, then the expression of 5-LO and MMP was assessed by immunocytochemical and Western blot analyses. The expression of 5-LO, MMP-2, and MMP-9 was stronger ($P < 0.01$) in macrophages isolated from diabetic plaques compared with from non-diabetic atherosclerotic plaques (Fig. 4A and B).

Enhanced LTB_4 biosynthesis in diabetic plaques macrophages

The amounts of LTB_4 were higher in homogenates of plaque tissue from diabetic patients compared with those from non-diabetic patients and that the differences were statistically significant, as assessed by ELISA. Furthermore, cultured macrophages isolated from diabetic lesions generated higher ($P < 0.01$) levels of LTB_4 as compared with those from non-diabetic lesions (Fig. 4C). LTB_4 is one of downstream products of the 5-LO pathway of arachidonic acid metabolism. Therefore, these results

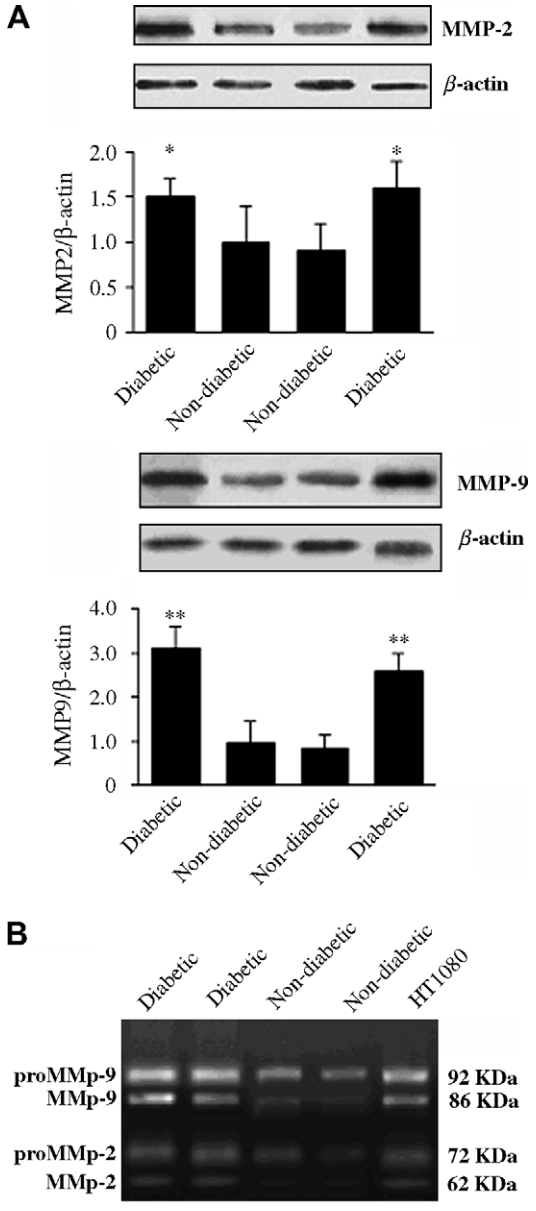

Fig. 3. The expression and activity of MMP-2 and MMP-9 were stronger in diabetic compared with non-diabetic atherosclerotic plaques. (A) The protein expression of MMP-2 and MMP-9 in diabetic and non-diabetic plaques was assessed by Western blot analysis using specific antibodies. The protein bands of MMP-2 and MMP-9 are indicated. β-Actin expression was used as an internal loading control. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the non-diabetic group. (B) The expression of the MMP-2 and MMP-9 activity was determined by zymographic analysis. HT1080 was used as a positive control with known gelatinolytic activity. The results are representative of studies that included 30 non-diabetic and 30 diabetic plaques.

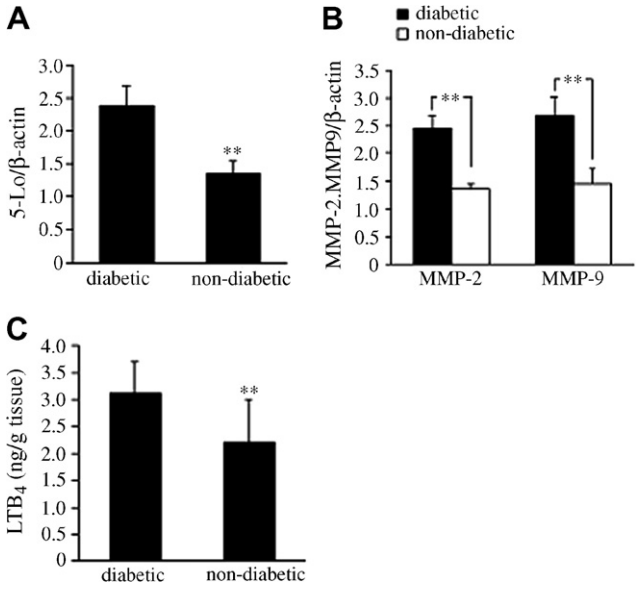

Fig. 4. Cultured macrophages isolated from non-diabetic and diabetic plaques recapitulate differences in 5-LO, MMPs, and LTB_4 levels observed *in vivo*. Quantitative analysis of 5-LO (A), MMP-2, and MMP-9 (B) levels was assessed by Western blot analysis followed by densitometric scanning of the immunoreactive bands. The quantitative densitometric scanning results were shown. The expression of 5-LO, MMP-2, and MMP-9 was stronger in macrophages isolated from diabetic plaques compared with from non-diabetic plaques. The levels of LTB_4 in non-diabetic and diabetic plaques were determined using ELISA (C). LTB_4 levels are higher in diabetic relative to non-diabetic atherosclerotic plaques. Data are presented as mean ± SD, $^{**}P < 0.01$ as compared with non-diabetic plaques.

demonstrate that elevated 5-LO protein expression in diabetic plaques is accompanied by elevated the arachidonic acid-metabolizing enzyme activity levels.

Discussion

Genetic studies have associated 5-LO and its accessory protein, 5-LO-activating protein, with cardiovascular disease encompassing atherosclerosis, myocardial infarction, stroke and aortic aneurysm [14]. Recent biologic and genetic findings implicate that the 5-LO pathway plays a critical role in atherosclerosis. The 5-LO pathway leads to the formation of 5(S)-hydroxyeicosatetraenoic acids (HETEs) and leukotrienes. Activation of the 5-LO pathway leads to the biosynthesis of proinflammatory leukotriene lipid mediators. Proinflammatory LTB_4 has been implicated in the pathogenesis of atherosclerosis. Previous studies have reported that inflammation plays a central role in the cascade of events that eventually results in plaque erosion and fissuring [15]. Recent studies suggest a strong association between overexpression of 5-LO pathway and atherosclerotic plaque instability in humans [2,16]. The 5-LO/LTB_4 signaling pathway has been linked to increase risk of acute ischemic events precipitated by MMPs-dependent processes that lead to plaque rupture, but very little is known regarding their role in diabetes.

In the present report, we provide evidence demonstrating for the first time to our knowledge that 5-LO was significantly overexpressed in diabetic compared with non-diabetic plaques, and it has been suggested that localized increase in 5-LO has the potential to cause the acute plaque disruption that precedes the onset of symptoms in both the coronary and cerebral circulations in diabetes. Because the arachidonic acid-metabolizing enzyme may

enhance synthesis and release of MMPs that can promote plaque rupture. In this study, concomitantly higher expression and activities of MMP-2 and MMP-9 were found in specimens obtained from diabetic patients compared with specimens obtained from non-diabetic patients. This finding is consistent with the report by Death et al. [17], in which the expression and activities of MMP-2 and MMP-9 were profoundly increased by high glucose culture of endothelial cells and macrophages. MMPs are the major matrix degrading enzymes and their activity has been correlated with clinical manifestations of unstable angina and plaque rupture [18,19]. In particular, expression of MMP-2 and MMP-9 has been shown within human atherosclerotic lesions and critically implicated in plaque rupture [20]. Thus, increased MMPs production may have important consequences for early events leading to plaque formation such as monocyte migration through the endothelium and also plaque rupture in diabetes; because these cells are known to preferentially accumulate in the vulnerable regions of atherosclerotic plaques.

In the present study, macrophages were more abundant in diabetic plaques, always outnumbered the lymphocytes, and represented the major source of 5-LO and MMPs. Furthermore, the site of inflammatory infiltration in diabetic plaques was always characterized by strong expression of 5-LO antigens on activated HLA-DR$^+$ inflammatory cells, which contrasted with the low expression of this protein elsewhere in the non-diabetic plaques. Thus, these data suggest the presence of more active inflammatory reaction in diabetic plaques. In fact, in agreement with the difference in 5-LO staining pattern, the histological milieu of the lesions appears different with regard to cellularity and presence of foam cells but not in the degree of vessel stenosis, suggesting that diabetic and non-diabetic lesions are different only with regard to inflammatory burden and that differences in plaque behavior stem from differences in the presence of as yet undetermined stimuli for selective expression of 5-LO capable of disrupting plaque stability.

Interestingly, this study provides new information that LTB_4 was significantly more abundant in diabetic plaques. Here, our finding that diabetic atherosclerotic plaques express elevated levels of both 5-LO and LTB_4 supports the hypothesis that LTB_4 may also be one of the key mediators of 5-LO-dependent plaque instability. LTB_4 is one of the downstream products of the 5-LO pathway and it is known to be involved in driving local acute inflammatory processes that precede and precipitate an acute thrombotic event. It has been well established that LTB_4 is a potent chemoattractant that promotes leukocyte adherence to the vascular endothelium. HG-induced adhesion of monocytes to endothelial cells could be mediated by the LO pathway. 5-LO-derived LTB_4 appears to influence early atherosclerotic events in mouse studies perhaps also by mediating monocyte adhesion and recruitment via monocyte chemoattractant protein-1 (MCP-1) [21–24]. The atherogenic activity of LTB_4 may, at least in part, be caused by its regulation on MCP-1 expression. LTB_4 strongly induces expression of MCP-1 through the BLT_1 pathway. Furthermore, this induction requires activation of ERK1/2 or JNK and may involve NF-κB activation. LTB_4 and MCP-1 amplify each other by a feed-forward loop leading to a greatly accelerated initiation and progression of the atherosclerotic lesions. Taken together, these results suggest an *in vivo* role for 5-LO pathway in diabetic atherosclerosis. It was concluded that the 5-LO pathway is important for mediating early vascular changes and inflammatory reactions in diabetes.

The present study support the hypothesis that diabetic patients may be at increased risk of developing unstable atherosclerotic plaques owing to elevated levels of both 5-LO and leukotrienes. It is tempting to speculate that the 5-LO pathway upregulation may promote MMP-induced atherosclerotic plaque disruption in diabetic patients. Because the 5-LO pathway can be upregulated by hyperglycemia, it is likely that it can augment diabetic atherosclerosis and vice versa, thereby setting off a vicious loop of events.

In summary, this study provides evidence that 5-LO expression and activity in macrophages are upregulated during hyperglycemia. We demonstrate that progression of atherosclerotic plaque toward rupture in diabetic patients is associated with upregulation of 5-LO protein and enzymatic activity in infiltrating macrophages, indicating a novel mechanism by which hyperglycemia could adversely affect the development of atherosclerotic lesions. Hence, these findings are potentially important from a fundamental standpoint because they enhance the mechanistic understanding of the crucial role of the 5-LO/LTB_4 signaling pathway in atherogenesis. From a practical standpoint, these findings provide a novel target for plaque stabilization and prevention of acute ischemic syndromes, particularly in the patients with diabetes.

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