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Development of Multiple Complications in Type 2 Diabetes Is Associated With the Increase of Multiple Markers of Chronic Inflammation

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Patients with type 2 diabetes (T2DM) are known at risk for developing cardiovascular disease (CVD), nephropathy, and cancer. We were interested to find out whether multiple markers associated with chronic inflammation are detectable in patients with T2DM and are increased in patients with T2DM who developed additional clinical complications. A sequence of multiple risk markers for atherogenesis, associated with chronic inflammation, was measured in patients with T2DM before and after the development of clinical complications. We found that multiple clinical complications frequently developed simultaneously in patients with T2DM. At the early stage of T2DM, only low levels and low percent elevations of multiple risk markers were detected. However, both the level and the percent elevation of these markers were found to increase with disease progression and the development of clinical complications. We believe that chronic inflammation not only contributes to the pathogenesis of T2DM but also continues to increase in T2DM patients who are developing additional clinical complications. It appears that these multiple markers are potentially useful not only for monitoring the progression of T2DM but also predicting the risk of developing macro- and microvascular disease, nephropathy, and cancer. J. Clin. Lab. Anal. 22:6–13, 2008.

Key words: type 2 diabetes; chronic inflammation; inflammation markers; nephropathy; cancer; macrovascular disease; microvascular disease

INTRODUCTION

Chronic systemic inflammation is now being recognized as the major causative factor for the pathogenesis of type 2 diabetes (T2DM) (1,2). It was also realized recently that the development and the propagation of several severe diseases could be found in T2DM patients, including macro- and microvascular disease (3,4) renal failure (5), and cancer (6) associated with chronic inflammation.

It is important to realize that chronic inflammation is systemic. As pointed out by Gabay and Kushner (7), new additional inflammation can be extended from any inflammatory disease to multiple new sites involving many organ systems, distant from the primary site of

Abbreviations: CVD, cardiovascular disease; CRP, C-reactive protein; ELISA, enzyme linked immunoassay; Hcy, homocysteine; PVD, peripheral vascular disease; uMA, urinary microalbumin; VCAM-1, vascular cell adhesion molecule; IL-6, interleukin 6; ICAM-1, intracellular adhesion molecule; T2DM, type 2 diabetes mellitus; UA, uric acid; uMA, urinary microalbumin.

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inflammation. For example, patients with cystic fibrosis, or asthma or chronic obstructive pulmonary disease (COPD) are at risk for developing additional inflammatory diseases such as diabetes (8), cardiovascular disease (CVD), and nephropathy (9) at new sites. We are also beginning to understand why patients with primary inflammatory diseases such as rheumatoid arthritis (10) and periodontal disease (11) are at risk of developing cardiovascular events. It is of special concern when life threatening diseases such as CVD, renal failure, and cancer are results of the dissemination from the local, primary inflammatory disease. Because patients with diabetic nephropathy are at risk for CVD, urinary microalbumin (uMA), an inflammatory risk marker associated with the severity of diabetic nephropathy, is now also considered as a risk marker for CVD (12). It should also be realized that the impact of chronic systemic inflammation is not limited to the development of these severe, fatal diseases. Multiple additional inflammatory diseases may also be developed at the same time but remain clinically silent (7).

In addition, to be the causative factor for the pathogenesis in T2DM (13,14) chronic systemic inflammation has also been found to be associated with the pathogenesis of CVD and diabetic nephropathy. Since nephropathy and macro- and microvascular disease are frequently developed in patients with T2DM, we have made an effort to investigate the relationship between chronic inflammation and T2DM, and also their clinical complications (15–17).

Sequential events are known to be associated with chronic inflammation (3,9). In coronary arteries, the early phase of atherogenesis begins with the appearance of proinflammatory cytokines, the expression of adhesion molecules, recruitment of leukocytes, entering of monocytes to the intima, and transformation of monocytes to macrophages. Because chronic inflammation is systemic, proinflammatory cytokines derived from chronic inflammation will spread and interact with hepatocytes, synthesizing acute inflammatory markers like C-reactive protein (CRP) and also cause leakage in kidney, producing microalbuminuria. At the same time, the myeloperoxidase (MPO) and reactive oxidative species released from the leukocytes will continue on downstream and generate oxidative and nitrosative stress. Because multiple markers corresponding to the sequential events of chronic inflammation in coronary artery have been identified (18) we decided to measure these multiple markers in T2DM patients both before and after they have developed clinical complications. In fact we have learned previously (19) that the progression of atherosclerosis from its onset to the advanced stage, such as unstable angina and myocardial infarction, is associated with a continuous increase of inflammation. We wondered whether the increase of inflammation is also associated with the progression from T2DM to its clinical complications. We were hoping that we might find the answer from the measurement of these multiple risk markers.

As anticipated, we have found in this investigation that increased inflammation, as shown in the increased level and percent elevation of multiple markers of chronic inflammation, was indeed associated with the disease progression of T2DM and with its further development of clinical complications. In the early stage of T2DM without any clinical complication we could only detect low levels and low percent elevations of all markers. However, increased levels of elevation, especially increased percent elevations of all markers, were found in T2DM when additional clinical complications have developed. We have also found that, in most cases, multiple inflammatory diseases were being developed simultaneously in T2DM. Because of genetic polymorphisms, it is conceivable that not everyone exposed to the same risk factor(s) will develop inflammatory disease. Consequently, these multiple markers will be useful for indicating the progression of inflammatory diseases and they are also useful to identify among individuals who may be at risk of developing T2DM and additional clinical complications.

MATERIALS AND METHODS

Subjects and Specimens

Specimens including blood and urine were collected from 662 patients with T2DM. A total of 129 of them had not yet developed clinical complication. There were 211 T2DM patients that had developed diabetic nephropathy (nephropathy was the only complication manifested). 31 developed cancer and 291 developed macro- and microvascular diseases. In most cases multiple complications had been developed. Some could still be clinically silent. All specimens were collected after overnight fasting at the Chang-Gung Memorial Hospital between November 2003 and August 2004. Average age of patients was 60 years old. The ratio of female to male was almost equal. Diagnosis of T2DM was based on National Cholesterol Education Program-Adult Treatment Panel III (NCEP ATPIII) criteria. In addition to their clinical history, all patients had gone through physical examination, electrocardiography (ECG), chest radiography, and ophthalmologic, neurological, foot, and retinoscopic examinations for the diagnosis of additional clinical complications. Most patients were taking medications such as drugs for hypertension, abnormal lipid profile, and hyperglycemia. No effort was made to identify those who were

undergoing treatment at the time of sampling. Those with microalbuminuria were based on uMA between 30 and 300 μg/mg creatinine. Those with albuminuria were based on the albumin/creatinine ratio >300 μg/mg. Subjects were excluded from the current study if they were on any of the following medications: glucocorticoids, antineoplastic agents, psychoactive agents, or bronchodilators.

Informed consent was obtained from all subjects included in this study and for blood drawing. The study protocol was approved by the Ethics Committee of Chang Gung Memorial Hospital. The protocol of the study was also approved by the ethics committee of Chang Gung Memorial Hospital.

Assays

All inflammation markers were measured by in-house developed enzyme-linked immunoassay (ELISA). All of these in-house kits have been compared with commercial kits with good to excellent correlation, including CRP (20), adhesion molecules (21,22), IL-6 (23), uMA (24), and homocysteine (Hcy) (25). Uric acid (UA) was determined by a Hitachi 7600-210 autoanalyzer (Hitachi, Tokyo, Japan).

RESULTS

Multiple Clinical Complications

In the early stage of T2DM, usually within the first five years of diabetes, T2DM patients are usually not associated with any additional clinical complications. Therefore, we found in this study that there were 129 of the 662 T2DM patients who had not yet developed clinical complications. Patients with longer T2DM history, such as 5–8.5 years, frequently developed nephropathy or CVD if not properly treated. As a consequence, 211 T2DM patients were found to have developed diabetic nephropathy (nephropathy was the only complication manifested).

There were 31 T2DM patients were found to have developed cancer and 291 developed macro- and microvascular diseases. We have also found that the development of additional clinical complications usually involved not a single but multiple inflammatory diseases. As described in "Subjects and Specimens," out of a total of 533 T2DM patients who had developed complications, 322 (approximately 60%) of them were found to have developed multiple complications. Many T2DM patients who developed CVD also had developed retinopathy or nephropathy or peripheral vascular disease. We also have noted that among those 37 patients who had developed cancer also had retinopathy or nephropathy or both. Many of those cancer patients had also developed macro- or/and microvascular disease. It appeared that some clinical complications remained clinically silent and were not detected. It was interesting to find that almost all T2DM patients with macro- and microvascular disease had microalbuminuria.

Higher Sensitivity With Inflammation Markers Than With HbA1c

We have identified 129 patients at the early stage of T2DM with an average diabetes history of 3.9 years and with good control of HbA1c (HbA1c <7%). None of them were associated with any additional complications such as nephropathy or CVD. None of them were taking any medication and none smoked. However, only moderate elevation of markers associated with chronic inflammation were detectable. As shown in Fig. 1, elevated markers, including proinflammatory cytokine (IL-6), acute inflammatory marker (CRP), adhesion molecules (VCAM-1, ICAM-1, and E-selectin), and the downstream systemic products such as uMA were all detectable in these T2DM patients. This finding indicated that a low level of inflammation existed in the early stage of T2DM as detected by the measurement of markers, even though the levels of HbA1c were normal. In other words, it appears that these multiple markers are more sensitive than that of HbA1c in detecting the presence of chronic inflammation.

It is interesting to note that uMA, a marker associated with cell leakage and impaired glomerular filtration rate of the kidney, was only slightly elevated in this group of patients at the early stage of T2DM. We also noted that the level and percent elevation of these multiple inflammatory markers were similar at the early stage of T2DM regardless of whether their HbA1c were >7% or <7% (data not shown). Again, our results suggest that HbA1c did not appear to be a sensitive marker to reflect the inflammatory condition in T2DM.

Inflammation Increases With the Development of Clinical Complications

As shown in Fig. 2, both the level and the percent elevation of these markers of chronic inflammation increased when additional complications developed. In other words, inflammation increased regardless whether the complication developed was related to nephropathy, cancer, or macro- and microvascular disease.

Among all these markers, uMA, one of the inflammation markers (26), showed the most dramatic increase upon the development of clinical complications. In regard to percent elevation, uMA raised from 19% to 100% in nephropathy, 55% in cancer, and 68% in macro- and microvascular disease. The level of uMA

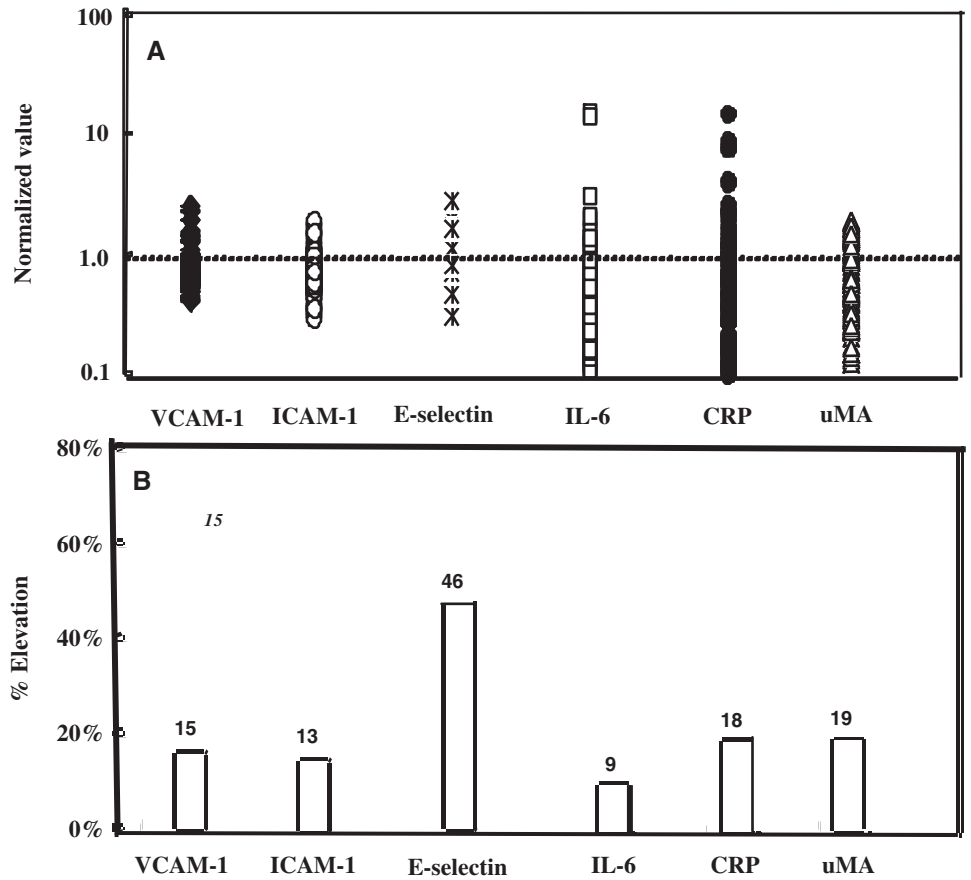

Fig. 1. Detection of elevation of multiple markers associated with chronic inflammation in patients with T2DM without clinical complications. These patients were in the early stage of T2DM before any additional complications had developed. They were all under good glucose control with HbA1c $<7\%$. **A:** Elevated levels of multiple markers were detectable in the early stage of T2DM. Normalized value is the determined level divided by the individual marker's upper cutoff. It allows comparison between markers with different units. Normalized value $= 1$ is represented by a dotted horizontal line. Values <1 are within normal range and >1 means elevated. **B:** Percent elevation found in all markers. Elevation of markers was based on the following cutoffs: IL-6, 6.4 pg/mL; hCRP, 3 mg/liter; VCAM-1, 769 ng/mL, uMA, 19 μg/mg creatinine; 8-OHdG, 55 ng/liter; UA, 8 mg/dL; and Hcy, 12 μmol/liter. Detection of elevation of these markers indicates that chronic inflammation is a more sensitive index to reflect the impact of hyperglycemia than the HbA1c.

also raised 20-fold in cancer and almost 60- and 70-fold in nephropathy and vascular diseases, respectively. All other inflammation markers also showed considerable increases coinciding with the development of clinical complications.

Recently, levels of UA and Hcy, in addition to being risk markers for CVD (25,26), have also been suggested to reflect inflammation based on different mechanisms (27,28). We added both markers to our panel in order to improve the sensitivity of detecting inflammation in a multiple marker format. Apparently Hcy is more sensitive than UA.

In this study, based on markers that we have measured, we could not find patterns distinctly different among various complications (Fig. 2) even though some involve primarily the epithelial cells (nephropathy, cancer) and some the endothelial cells (vascular diseases). In fact, the patterns of both the mean and the percent elevation were very similar to each other regardless what complication was developed. It is most likely that multiple complications were frequently being developed in T2DM, and development always involve multiple types of cells. In addition, no specific pattern has so far been identified with the development of any specific type of inflammatory disease.

Inflammation Increases With Disease Progression

In atherosclerosis, a continuous increase of inflammation is associated with the progression from the onset of atherosclerosis to stable angina, unstable angina, and eventually to myocardial infarction (18). Here we found that inflammation not only increased in T2DM during the development of additional complications but

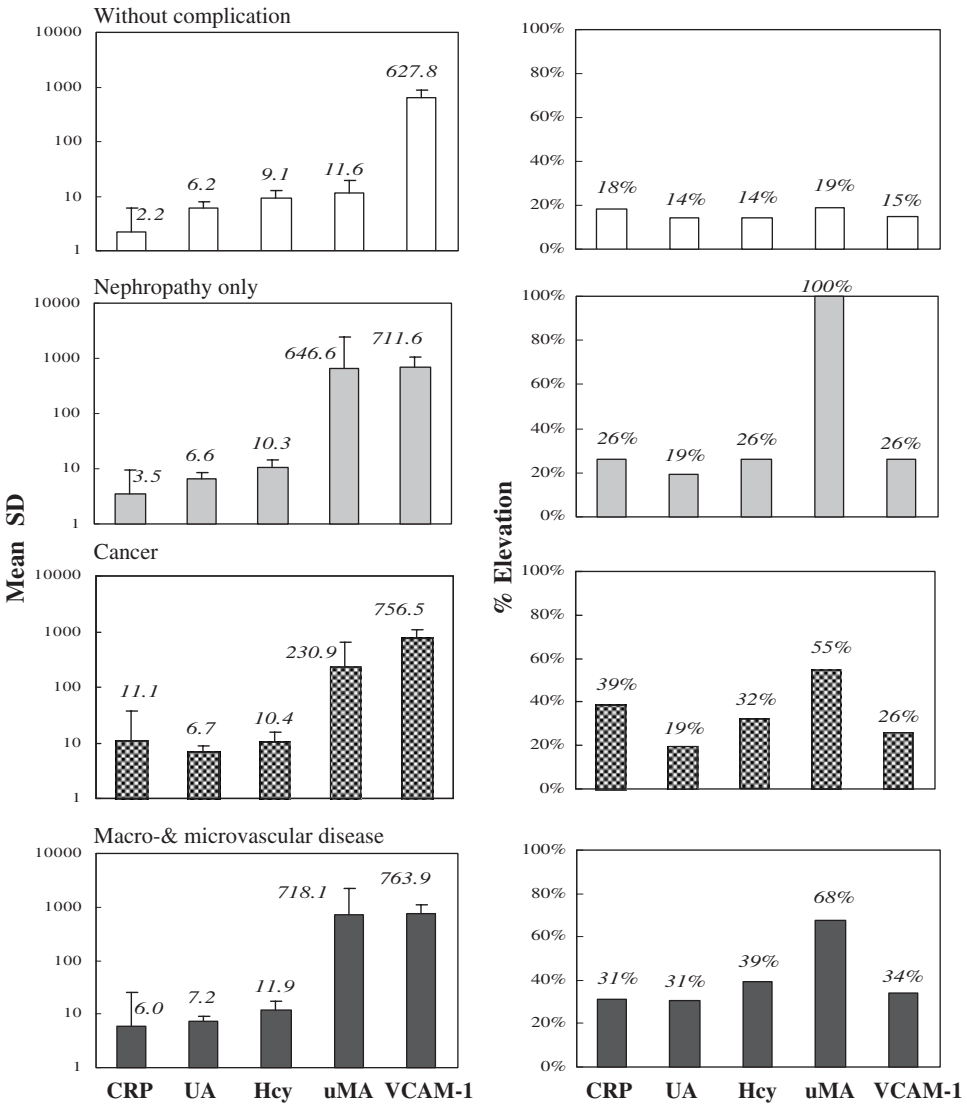

Fig. 2. Increase of inflammation in T2DM patients with the development of additional clinical complications. Increase of levels of all markers associated with chronic inflammation (shown as mean $\pm$ SD) was found in T2DM patients who developed either nephropathy (the only complication that was manifested), cancer (other complications may exist), or macro- and microvascular disease (there were usually multiple complications coexisting) on the left side of the figures. Among all markers uMA shows a most dramatic increase. Values of the mean are listed on top of the bar. Increase of the percent elevation was also found with all markers on the right side of the figures. Again, the largest increase was found with uMA. However, significantly higher increase was found in all markers when T2DM patients developed macro- and microvascular disease.

also during the course of the disease. We chose to measure inflammation markers following the disease progression of diabetic nephropathy because the progression of diabetic nephropathy can be conveniently monitored in terms of the concentration of uMA. As shown in Fig. 3, both the level and the percent elevation of all the markers measured increased with the increase of uMA from $<30\,\mu g/mg$ (onset of nephropathy) to $300\,\mu g/mg$ (onset of albuminuria). It appears that, in addition to uMA, both VCAM-1 and Hcy are also sensitive markers reflecting the disease progression in diabetic nephropathy.

Advantages of Measuring Multiple Markers

Even though these multiple markers that we measured in this study correspond to sequential of events of chronic inflammation, the sensitivity of most of these single markers is relatively low. However, these markers appear to complement to each other so that if measured simultaneously, the sensitivity will increase. In fact, the advantage of measuring multimarkers has also been recognized by Morrow and Braunwald (29) for the diagnosis of acute coronary syndromes. As illustrated in Fig. 4, measurement of VCAM-1, uMA, CRP, Hcy, and

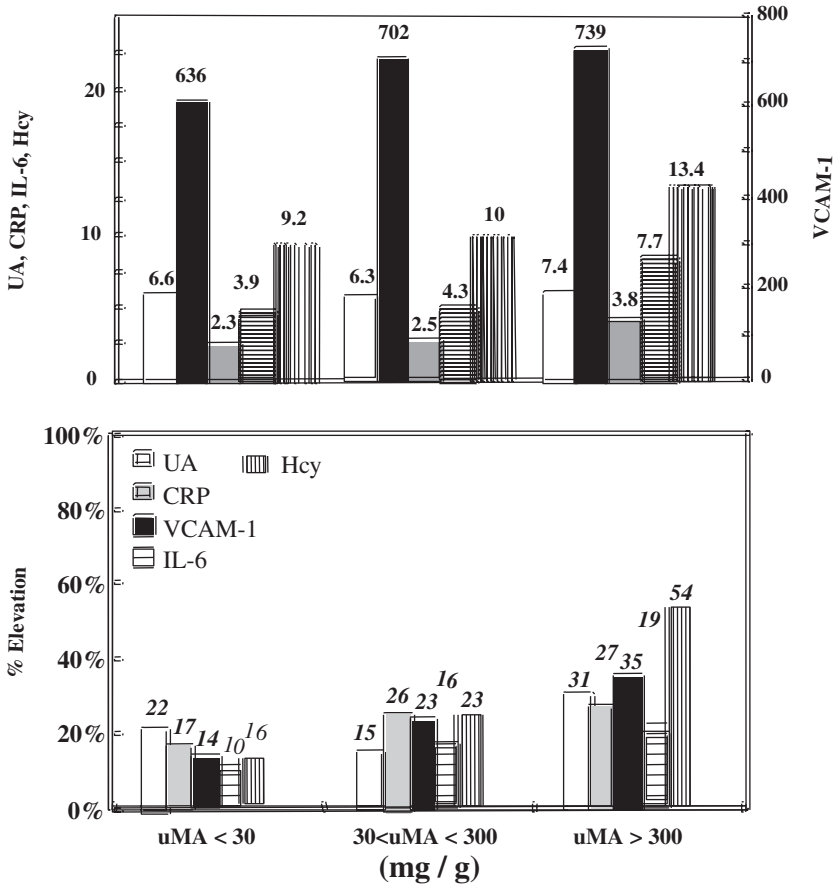

Fig. 3. Continuous increase of inflammation during disease progression of diabetic nephropathy. Disease progression of diabetic nephropathy was conveniently divided into three stages based on the value of uMA. Apparently both levels and percent elevation of all markers continue to increase with the disease progression except that of UA. The reason is not clear at the present time. Both VCAM-1 and Hcy appeared to be the next more sensitive markers, next to uMA, to response to the disease progression. Levels (mean) or various markers are also listed on the top of each bar (see legend of Fig. 1 for units).

UA at the same time have improved the percent elevation in all three different disease cohorts. It also appears that the combination of just VCAM-1 and uMA provided almost close to the maximal sensitivity. It should be noted that the maximal sensitivity in any combination of multiple markers may vary from individual to individual and may be different in different disease groups. More detailed studies may be required to determine the most optimal combination of markers for any specific disease.

DISCUSSION

Apparently the impact of chronic inflammation is truly systemic and widespread. It is important to realize that chronic inflammation not only plays an important role in the pathogenesis of T2DM, it will also spread to other sites of the body and initiate new inflammatory disease at distant sites. Apparently the impact of many inflammatory risk factors is also systemic (18). For example, the impact of abdominal fat, hyperglycemia, etc. can reach multiple sites of the body simultaneously. Moreover, because of the systemic nature of chronic inflammation, the dissemination from a local inflammatory disease will not limited to a single new inflammatory disease but multiple clinical complications.

It is not clear what determines the new site(s) that will be affected from a local inflammation. We can speculate with what we know at the present time but there is more to be learned. Macro- and microvascular disease and nephropathy are most frequently detected complications in T2DM; therefore, it is likely that blood vessel and kidney are most accessible to be affected by chronic inflammation derived from any primary inflammatory disease. The less frequent complication of cancer could be due to the additional multiple mutations of growth genes required for tumorigenesis. Unfortunately all these new complications frequently lead to fatal consequences.

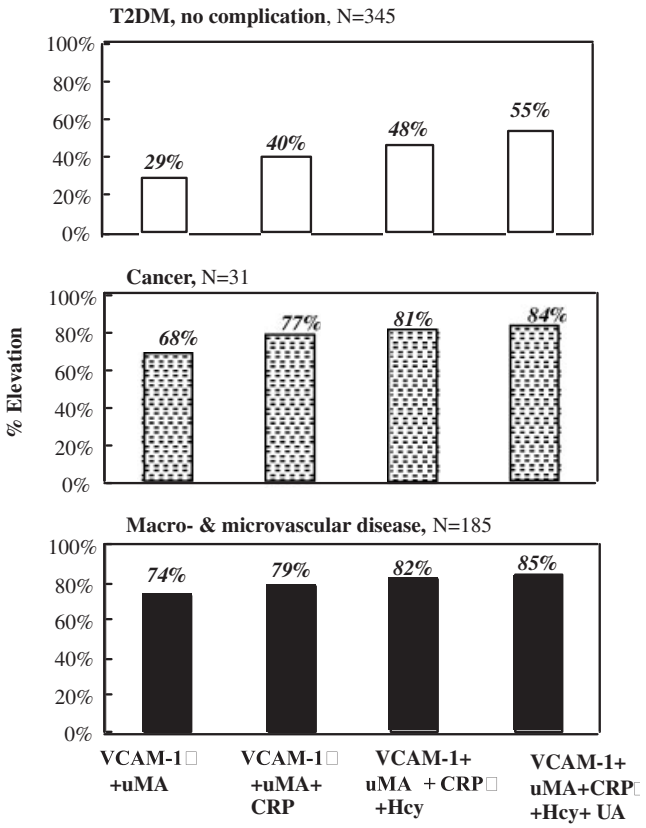

Fig. 4. Increased sensitivity by monitoring multiple markers. Monitoring multiple markers apparently provides increased sensitivity over any single marker. Here uMA shows the highest sensitivity as a single marker; however, higher sensitivity will be obtained if multiple markers are measured simultaneously in all three groups of patients.

We found that it is useful to monitor the progression of the inflammation associated with any inflammatory disease by measuring multiple markers corresponding to the sequence of events of chronic systemic inflammation (18). It is most likely that regardless of where (which organ or tissue) the inflammation take place, the overall sequence of events may always include cell injury, production of proinflammatory cytokines, production of acute reactant by hepatocytes, recruitment of leukocytes, and the subsequent oxidative and nitrosative stress. Consequently, it is always possible to detect elevation of various products of chronic inflammation such as IL-6, CRP, adhesion molecules, uMA, UA, and Hcy. These markers not only indicate the presence and reflect the progression of chronic inflammation but also alert us the risk of the development of other complications. It should be noted that the acute inflammatory response detected by the elevation of IL-6 and CRP, for example, is the early phase of chronic inflammation. Subsequent events of chronic inflammation are expression of adhesion molecules, appearance of microalbuminuria, and oxidative and nitrosative stress. UA and Hcy also reflect the overall inflammation based on different mechanisms. In fact, as pointed out by Hayden and Tyagi (30), Hcy and UA are, like CRP and oxidized low-density lipoproteins (LDL), capable of producing injurious stimuli to the endothelium, the arterial vessel wall, and capillaries, causing inflammation and vascular disease (31). In this study we have found that low-grade inflammation is present during the early stage of T2DM. The inflammation never stops. The inflammation continues to increase during the progression of the disease and continues to be enhanced during the spread and the development of additional complications. Measuring multiple markers of chronic systemic inflammation apparently is helpful for monitoring the disease progression and the extent of dissemination.

It should be realized that many products of early inflammation events are proinflammatory. CRP, for example, is proinflammatory (32,33). A vicious circle of inflammation may therefore exist, causing the inflammation to intensify with the progression of the inflammatory diseases. This explains why the progression of atherosclerosis is associated with a further increase of inflammation. Conceivably, for treatment of any disease involving chronic inflammation, it is important to identify and remove the inflammatory risk factor(s), but equally important is to reduce inflammation to prevent progression of the disease and additional clinical complications. It should be mentioned that lifestyle change, nutritional modification, or taking antiinflammatory medication would also help reduce inflammation (34).

We believe that these multiple markers not only are useful to predict risk of atherogenesis but would also provide indication of tissue damages systemically. For example, elevation of uMA is a risk signal for CVD but also alerts us to the impairment of renal glomerular filtration (12). These multiple markers can also be used to monitor treatment, lifestyle change, and nutritional modification, all of which impact chronic inflammation.

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