Original Article

Midkine Levels and its Relationship with Atherosclerotic Risk Factors in Essential Hypertensive Patients

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ABSTRACT

Background and Objectives: Hypertension (HT) is one of the risk factors associated with atherosclerosis. Midkine (MK) plays a role as a growth factor in various biologic and pathologic events. In some reports, MK expression has been shown to be linked with vascular smooth muscle proliferation and neo-angiogenesis in atherosclerotic vessels. The aim was to research relationship of MK serum levels with some atherosclerotic risk factors in hypertensive patients. **Methodology:** This study examined 60 patients with essential HT and 30 healthy controls. Serum biochemistry, including lipid profile, MK, Vitamin B_{12}, C-reactive protein, zinc and copper levels were obtained. **Results:** MK levels of the HT patients were significantly higher than the control group (24.8 ± 6.8 ng/mL vs. 18.39 ± 5.6 ng/mL, respectively, $P < 0.01$). Lipid profile parameters such as total cholesterol, triglyceride, low-density lipoprotein (LDL) were also significantly higher in HT patients ($P < 0.021$, $P < 0.01$, and $P < 0.01$, respectively). Zinc levels were 179.13 ± 34.06 µg/dL and 172.55 ± 45.47µg/dL in the HT and control group, respectively. Serum MK levels were positively correlated with diastolic ($r = 0.288$, $P < 0.05$) and systolic blood pressures ($r = 0.390$, $P < 0.002$), and also with serum total cholesterol ($r = 0.406$, $P < 0.002$) and LDL cholesterol ($r = 0.318$, $P < 0.015$) levels. Furthermore MK was also negatively correlated with zinc and Vitamin B_{12} levels ($r = -0.298$, $P < 0.023$, $r = -0.334$, $P < 0.027$, respectively). **Conclusion:** This study has demonstrated an important association between increased serum MK levels and risk factors of atherosclerosis such as HT, increased total and LDL cholesterol.

KEYWORDS: *Atherosclerosis, cholesterol, hypertension, midkine*

INTRODUCTION

Essential hypertension (HT), defined as systolic/diastolic blood pressure (BP) of 140/90 mmHg or greater, is a complex and multifactorial disease.[1] It is one of the well-known risk factors of cardiovascular disease (CVD) and stroke. Efficient treatment of HT markedly reduces premature deaths due to CVD.[2] Multiple and complex interactions of some vascular effectors, such as catecholamines, the renin-angiotensin system (RAS), oxidative stress,[3] nitric oxide (NO), vascular endothelial growth factor, endothelin-1, and inflammatory cytokines[4] are involved in the pathophysiology of systemic HT.[5,6] In many newly diagnosed hypertensive patients, at least one lipid abnormality is usually found.[7]

Atherosclerosis is a complex disease caused by the accumulation of deposits known as atheromas or plaques in the intima of blood vessels, leading to narrowing of arteries.[8] It is characterized by subendothelial lipid accumulation, endothelial dysfunction, recruitment of monocytes into the endothelium; migration and differentiation of smooth muscle cells (SMCs) to the intima and eventually the formation of atherosclerotic plague under the

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effects of local flow hemodynamics and inflammatory mechanisms.[9]

It can be specified as a chronic inflammatory disease of large-sized and medium-sized arteries.[10] Hypercholesterolemia, free radicals from cigarette smoking, diabetes mellitus, and HT are well-known risk factors for development and progression of atherosclerosis.[11] The coexistence of both HT and dyslipidemia is common.[12] Atherosclerosis is related to imbalanced lipid metabolism and a maladaptive immune response of arterial walls.[13] Underlying mechanisms of the atherosclerotic process include endothelial cell dysfunction, inflammatory and anti-inflammatory cytokines, regional hypoxia, neo-angiogenesis, and increased cell turnover with proliferation and apoptosis.

Midkine (MK), a heparin-binding growth factor with a molecular weight of 13 kDa, has various biological functions such as the growth of fibroblasts,[14] survival of embryonic neurons,[15] and migration of inflammatory cells[16] which are elicited through extracellular signal-regulated kinase and AKT activation.[15] MK is expressed by endothelial cells.[17] Hobo *et al.* reported that MK up-regulate pulmonary angiotensin-converting enzyme (ACE) and so enhance HT in the 5/6 renal ablation model of chronic kidney disease (CKD).[18] On the other hand, it has been shown that MK regulated cytochrome P450-derived epoxyeicosatrienoic acids (EETs)' activity, a regulator of the endothelial vasodilators release, and thereby BP.[19]

Recent studies point to the role of MK in chronic and acute inflammatory processes.[10,13] MK has also been associated with the inflammatory diseases such as rheumatoid arthritis and osteoarthritis,[20] diabetic nephropathy,[21] atherosclerosis,[22,23] multiple sclerosis,[24] Crohn's disease,[25] and ulcerative colitis.[26] It has been reported that MK promoted the migration of leukocytes, induced the chemokines and suppressed the regulatory T cells in the inflammatory process.[27-29] Atherosclerosis has been shown to be related with MK in animal models. It has been demonstrated that MK is expressed at very low levels in the healthy arteries and veins.[23] However, its expression was increased in the endothelium of various animal models.[17,23] Considering the inflammatory nature of atherosclerosis, MK appears to play a prominent role in the pathogenesis of atherosclerosis.

Consequently, the objectives of this study were to investigate the effects of MK on HT and also to evaluate the relationship between MK and risk factors of atherosclerosis such as total cholesterol, low-density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol, triglyceride, high sensitive C-reactive protein (hs-CRP), gender, age, Body mass index (BMI). We also searched its association with Vitamin B_{12}, folic acid, and some trace elements such as zinc and copper to find underlying mechanisms of MK action.

MATERIALS AND METHODS

Patients

This study was a cross-sectional study and included a total of 90 participants, including 60 essential hypertensive subjects (53.83 ± 7.71 years old) and 30 healthy controls (51.18 ± 6.80 years old) without symptoms and signs suggestive of HT and with no family history of the hypertensive disease. HT was defined as systolic BP (SBP) ≥140 mm Hg, diastolic BP (DBP) ≥90 mm Hg, current treatment with antihypertensive medication, or a self-reported diagnosis of HT. BP of the patients was measured on the right arm following 10 min rest in the seated position using a manual sphygmomanometer. Measurements were performed twice at 5 min intervals, and the average was calculated for each individual. Exclusion criteria included secondary HT, diabetes, cancer, recent acute infection, severe cardiac, liver or kidney dysfunction, and cerebrovascular disease. All the participants underwent a standardized clinical assessment, which included a medical history and physical as well as a neurological examination. Hypertensive patients received treatment as necessary. Informed consent was obtained from all participants before the study. This clinical investigation was approved by the local ethics committee.

Blood collection

Following an overnight fast, blood samples were collected from a vein in the antecubital fossa without venous occlusion, and the samples were immediately centrifuged. Serum was separated and then stored at −80°C until biochemical analysis.

Methodology

Glucose, urea, and creatinine levels were measured using assay kit from Cobas, Roche Diagnostics. Estimation of lipid profile (total cholesterol, HDL- cholesterol and triglyceride levels) was carried out, using a clinical chemistry auto analyzer (Cobas, Roche Diagnostics). LDL-cholesterol was calculated according to Friedewald's (1972) Formula.[30] Vitamin B_{12}, folic acid, free thyroxin, thyroid stimulating hormone, and insulin were determined using electrochemiluminescence immunoassay using commercial kits from Roche (Cobas E411, Roche, Japan).

Midkine assay

Serum MK levels were measured using Biovendor ELISA Kit, based on the competition principle and

microtiter plate (MTPL) separation. Interassay and intraassay CV percentages were <8% and <5%, respectively (BioVendor – Laboratorní Medicína a.s.; Brno, Czech Republic).

Zinc and copper assay

Zn and Cu levels of serum were determined by an atomic absorption spectrophotometer with flame (Shimadzu AA-6800) at 213.9 nm for zinc and 324.8 nm for copper. The results were calculated as micrograms per deciliter for serum. The standards were freshly prepared from standard stock metal solutions, Titrisol 1.000 ± 0.002 mg/L (Merck Darmstadt, Germany) immediately before analysis and were used for initial calibration for each element analysis. These solutions were also used as internal quality standards. Hollow cathode lamp and background correction (with deuterium lamp) modes were selected for element analysis. For each analysis deionized water was used to clean the chamber and the zero control. Stock standard solutions of zinc and copper were used for control which were tested every 90 measured samples to ensure the reliability of the measurement.

Statistical analysis

All data were analyzed with the use of the Statistical Package for the Social Sciences for Windows software (Version 15.0 SPSS, Chicago, IL, USA). Data were presented as mean and SD (±) or percentage (%). The differences between groups were identified by using unpaired *t*-tests for parametric and Mann–Whitney U-test for nonparametric data. Correlations between the variables were evaluated using Pearson correlation coefficient. Statistical significance was defined as $P<0.05$.

RESULTS

The analyzed parameters and statistical analysis of the results for HT patients and healthy controls are given in Table 1. BMI was significantly higher ($P < 0.001$) in HT patients compared to controls. In the HT parameters, SBP ($P < 0.001$) and DBP ($P < 0.001$) were significantly higher in hypertensive patients compared to the control group.

MK levels of the HT patients (24.8 ± 6.8 ng/mL) were significantly higher compared to the control group (18.39 ± 5.6 ng/mL) ($P < 0.002$) [Figure 1]. Lipid profile parameters such as total cholesterol, triglyceride, and LDL cholesterol were also significantly higher in HT patients ($P < 0.021$, $P < 0.003$, and $P < 0.01$, respectively). HDL cholesterol levels did not differ statistically between two groups. hsCRP was significantly higher in the hypertensive patients than controls ($P < 0.001$).

Table 1: Clinical assessments and laboratory findings in hypertension and control groups

	Control	Hypertension group
n	30	60
Age (years)	51.18±6.80	53.83±7.71
BMI (kg/m²)	27.14±4.04	32.16±4.8***
WHR (Gw/Gh)	0.86±0.07	0.86±0.26
SBP (mmHg)	114 (90-130)	150 (125-200)***
DBP (mmHg)	80 (60-90)	100 (95-150)***
Hypertension time (years)	-	6.6 (4-32)
Glucose (mg/dL)	90.31±11.13	107.18±14.26
Triglyceride (mg/dL)	130.27±85.51	181.20±106.67**
Total cholesterol (mg/dL)	199.31±38.09	223.84±48.76*
HDL-cholesterol (mg/dL)	47 (29.00-65.00)	47 (32-74)
LDL-cholesterol (mg/dL)	108.61±15.93	141.44±40.09**
Creatinine (mg/dL)	0.73±0.16	0.86±0.48
Urea (mg/dL)	22.66±7.2	25.9±8.54
TSH (mIU/mL)	2.01 (0.91-4.17)	1.78 (0.61-5.56)
Free T4 (ng/dL)	1.04 (0.76-1.53)	1.17 (0.68-1.41)
MK (ng/mL)	18.39±5.6	24.8±6.8**
CRP (mg/L)	2.43±0.99	5.86±2.89***
Vitamin B12 (pg/mL)	263.37±76.69	211.89±52.91**
Folic acid (ng/mL)	8.13±1.68	6.62±2.17**
Zinc (µg/dL)	172.55±45.47	179.13±34.06
Copper (µg/dL)	167.85±43.57	152.60±43.50

*$P<0.05$, **$P<0.01$, ***$P<0.001$. SBP, DBP, HDL-cholesterol, TSH and free T4 were presented as median (interquartile range), other characteristics were presented as means±SD. BMI=Body mass index; WHR=Waist to hip ratio; SBP=Systolic blood pressure; DBP=Diastolic blood pressure; HDL=High-density lipoprotein; LDL=Low-density lipoprotein; CRP=C-reactive protein; TSH=Thyroid stimulating hormone; Free T4=Free thyroxine; SD=Standard deviation; MK=Midkine

Table 2: Clinical assessments and laboratory findings according with in hypertension time groups

	Hypertension time		
	<5 years (*n*=17)	5-10 years (*n*=24)	>10 years (*n*=19)
Age (years)	50.78±6.99	58.22±6.54	54.27±7.56
MK (ng/mL)	22.99±7.25	24.33±6.86	29.62±9.65[a],*
Vitamin B12 (pg/mL)	271.38±86.5	225.94±61.12	217.8±54.74[a],*
Folic acid (ng/mL)	6.51±2.33	6.87±2.39	6.52±1.62
Zinc (µg/dL)	179.04±47.94	168.17±44.93	152.95±40.53[a],*

*$P<0.05$; [a]<5 years versus >10 years. MK=Midkine

Vitamin B_{12} (pg/mL) and folic acid (ng/ml) levels were 263.37 ± 76.69 and 8.13 ± 1.68, respectively, in control group and 211.89 ± 52.91 and 6.62 ± 2.17, respectively, in the HT group. Both of these two vitamins were statistically lower in HT patients compared to controls ($P < 0.009$ for Vitamin B_{12} and $P < 0.002$ for folic acid).

Zinc levels were 179.13 ± 34.06 µg/dL in HT group and 172.55 ± 45.47 µg/dL in control patients. In the same way as the zinc, copper levels were not significantly

Table 3: Pearson correlation coefficient Midkine and biochemical parameters in patient group

Parameters	Correlation coefficient (r)	P
BMI	0.266	0.043
SBP	0.390	0.002
DBP	0.288	0.046
Total cholesterol	0.406	0.002
LDL cholesterol	0.318	0.015
Vitamin B12	−0.334	0.027
Zinc	−0.298	0.023

BMI=Body mass index; SBP=Systolic blood pressure; DBP=Diastolic blood pressure; LDL=Low-density lipoprotein
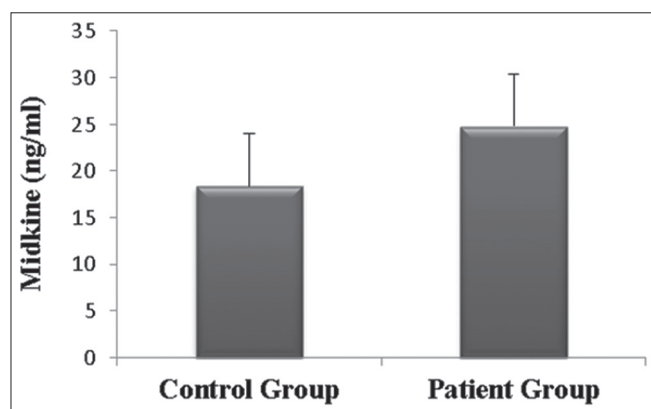
Figure 1: Midkine levels in hypertensive and control groups

changed in hypertensive patients [Table 1] compared to control. However, zinc serum levels were significantly decreased in HT patients for longer than 10 years, as given below.

We also examined the change in MK levels and some other parameters according to the duration of HT. Hypertensive patients were classified according to the time of first diagnosis of HT: 0–5 years ($n = 17$), 5–10 years ($n = 24$), and > 10 years ($n = 19$). In the group of patients with > 10 years HT, MK levels were found to be significantly higher than the 0–5 years HT patients ($P < 0.05$). In contrast, Vitamin B$_{12}$ ($P < 0.05$) and zinc ($P < 0.05$) levels were significantly lower in >10 years HT patients compared to 0–5 years HT patients [Table 2]. MK, Vitamin B$_{12}$ and zinc serum levels were changed consistent with the duration of HT.

A positive correlation was found between MK levels and BMI ($r = 0.266$, $P < 0.043$) diastolic ($r = 0.288$, $P < 0.05$), SBP ($r = 0.390$, $P < 0.002$), total cholesterol ($r = 0.406$, $P < 0.002$), and LDL cholesterol ($r = 0.318$, $P < 0.015$). Furthermore, a significant negative correlation was observed between MK levels and zinc levels and Vitamin B$_{12}$ levels ($r = −0.298$, $P < 0.023$, $r= −0.334$, $P < 0.027$, respectively) [Table 3].

DISCUSSION

In this study, we found that MK levels were increased in hypertensive patients. Lipid profile parameters such as total cholesterol, triglyceride, LDL, and CRP were also higher in these patients. Vitamin B$_{12}$ and folic acid levels were decreased in the HT group. The trace element zinc serum levels were significantly decreased in HT patients >10 years. MK levels were positively correlated with BMI, diastolic, SBP, total, and LDL cholesterol. Furthermore, MK was negatively correlated with zinc and Vitamin B$_{12}$ levels. The association of MK with HT and risk factors of atherosclerosis in human was shown for the first time in our study.

Essential HT is a complex and multifactorial disease. It is one of the well-known risk factors of CVD and stroke. Multiple and complex interactions of some vascular effectors, such as catecholamines, the RAS, oxidative stress, NO, vascular endothelial growth factor, endothelin-1, and inflammatory cytokines are involved in the pathophysiology of systemic HT.[3-6] As a growth factor, MK has various biological functions in inflammation, angiogenesis, immunity, pathogenesis of cancer and some other diseases that were extensively reviewed recently.[31-34] In the literature, it has been reviewed that MK has a role in etiopathogenesis of HT.[5,10] Horiba et al. showed that MK is expressed by endothelial cells.[17] Hobo et al. reported that MK upregulated the pulmonary ACE in the 5/6 renal ablation model of chronic kidney disease, by this way enhanced BP.[18] MK regulates HT through RAS activation. In the remnant kidney model as a model of advanced renal injury, Hobo et al. showed that SBP and mean BP were significantly higher in the Mdk+/+ mice than in the Mdk−/− mice.[18] A study by Sato et al. found that MK/EET (cytochrome P450-derived epoxyeicosatrienoic acids) pathway participated in BP control by regulating EDHF (endothelium-derived hyperpolarizing factor) release. This pathway was independent from RAS.[19]

These results in the literature showing association of MK with HT were mostly demonstrated in experimental animal models. In this study, the association of MK with HT in human was shown for the first time. HT is a well-known risk factor for cardiovascular diseases due to atherosclerotic complications. In this pathophysiological circle, MK may play a role as a common denominator for vessel and tissue alterations. In our hypertensive patients, well-known risk factors of atherosclerosis such as HT, BMI, total cholesterol, triglyceride, LDL cholesterol, and high-sensitivity hs-CRP were significantly higher than controls. Atherosclerosis is characterized by subendothelial lipid accumulation, endothelial dysfunction, recruitment of monocytes

into the endothelium; migration and differentiation of SMCs to the intima and eventually the formation of the atherosclerotic plaque.[9] Hence, underlying mechanisms of this atherosclerotic process include endothelial cell dysfunction, inflammatory and anti-inflammatory cytokines, regional hypoxia, neoangiogenesis, and cell turnover with proliferation and apoptosis.

MK has various biological functions such as the growth of fibroblasts, and migration of inflammatory cells.[14,16] Recent studies pointed to the role of MK in acute and chronic inflammatory processes and diseases.[10,13,20,21,25,26] It has been reported that MK promoted the migration of leukocytes, induced the chemokines and suppressed the regulatory T cells in the inflammatory process.[27-29] Atherosclerosis has been shown to be related to MK in animal models. MK is expressed at very low levels in the healthy arteries and veins.[23] However, increased MK expression due to the injury of the endothelium was shown in the various animal models. Narita *et al.* showed that macrophages are the major source of MK in the atherosclerotic neointima of hypercholesterolemic rabbits.[23] MK is an essential factor for neointimal hyperplasia.[22] Horiba *et al.* reported that neointima formation reduced in carotid artery ligated Mdk-/- mice, and this effect was reversed by systemic MK administration.[23] Salaru *et al.* showed circulating MK serum levels in patients with peripheral artery disease inversely correlated with angiotensin and endothelin receptor autoantibody titers.[35] They have also searched the relationship of serum MK levels with the traditional risk factors of atherosclerosis such as age, sex, obesity, smoking, HT, high cholesterol levels, and diabetes mellitus. However, they could not find any correlations between MK serum levels and any of these risk factors. Whereas, in this study, we demonstrated that there were positive correlations between MK serum levels and BMI, diastolic, SBPs, total cholesterol, and LDL cholesterol. In our study, we selected patients with essential HT without any complications of infectious or inflammatory diseases. Their patients have been operated for severe peripheral artery disease suggesting that acute inflammation due to tissue damage was in play. Muramatsu *et al.* emphasized this point by defining MK as a double-edged sword,[36] with beneficial and harmful effects in the tissue. The hallmark of atherosclerosis is chronic inflammation in the vessel walls. The effects of MK on the migration of inflammatory leukocytes, expression of pro-inflammatory cytokines, macrophage infiltration, and migration of SMCs and the propagation of neointima formation may result in vascular stenosis and atherosclerosis. Considering the inflammatory nature of atherosclerosis, MK appears to play a prominent role in the etiopathogenesis of atherosclerosis. It seems to be a common denominator for pathophysiological changes seen in atherosclerosis.

A number of studies have reported associations between trace metals and atherosclerosis. It was reported that zinc deficiency is associated with the development of atherosclerosis.[37-39] In this study, zinc and copper serum levels were not different in hypertensive patients compared to controls. However, serum levels of zinc were significantly decreased in HT patients >10 years. We also found a significant negative correlation between serum zinc and MK levels. All these findings are pointing to the role of zinc associated with MK in this disease concept. This complex intermolecular network of zinc, MK and other inflammatory modulators should be elucidated with further studies.

This study has some limitations and could be supported with further research with respect to the following points primarily. The study was cross-sectional and had a relatively small sample size. The cross-sectional design does not permit to draw any conclusions on a causal relationship between elevated MK levels and incidence of atherosclerosis and HT. It is necessary to confirm the findings in the study with a larger sample size and with the suggested edits to the measurement frequency.

CONCLUSION

Serum MK levels were increased in hypertensive patients. Lipid profile parameters such as total cholesterol, triglyceride, LDL, and CRP were also higher in the same patients group. Vitamin B$_{12}$ and folic acid levels were decreased in the HT group. The trace element, zinc serum levels were significantly decreased by the time. Furthermore, MK levels positively correlated with diastolic, SBP, total and LDL cholesterol; and negatively correlated with zinc, Vitamin B$_{12}$ levels. These results suggest that increased MK levels can be related to atherosclerosis in HT patients. This association of MK with HT and risk factors of atherosclerosis in human was shown for the first time. The study results emphasize the need for further research on the role of MK levels in the etiology and progression of atherosclerosis and HT.

Conflicts of interest

There are no conflicts of interest.

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