compound heterozygous control (Fig. 1C, middle and bottom panels). These results thus confirm that patient 1, her mother, and patient 2 are heterozygous for the novel HKαα allele, fully explaining the previous Southern, multiplex-PCR, and pedigree analysis (Fig. 1D) results.

Because the HKαα allele contains neither deletion nor triplication, carriers of this novel allele are unlikely to suffer any deleterious effects; however, the existence of deletions and triplications detect unequal crossover junctions; therefore, for DNA samples that are positive for both the −α3.7 and αααanti-4.2 junction fragments by PCR analysis, it is no longer possible to definitively make a conclusive diagnosis of compound heterozygous −α3.7 and αααanti-4.2. Further confirmation is necessary, either by Southern analysis or the 2-round nested PCR analysis described in this report, or by pedigree analysis of parents and siblings, if available.

Further confirmatory analysis is required only when the PCR results are positive for both the −α3.7 and αααanti-4.2 junction fragments. Given the rarity of the −α3.7/αααanti-4.2 compound heterozygous genotype, as well as the presumed rarity of the HKαα allele, such additional analyses are unlikely to be necessary on most occasions, and PCR-based testing for single gene deletions and triplications are likely to continue being widely used in diagnostic laboratories.

The novel HKαα allele documented in this report could have originated through one of several mechanisms. The first involves a nonreciprocal gene conversion event (see Fig. 2 in the online Data Supplement). The second involves a simultaneous double crossover between misaligned X and Z boxes (see Fig. 3 in the online Data Supplement). More likely, we believe, the HKαα allele originated via an intermediate recombinant allele such as the −α3.7 or αααanti-4.2 allele, or both. Three possibilities are likely under this assumption. The first involves unequal crossover between the Z1 box of a wild-type allele and the Z2 box of an αααanti-4.2 allele to generate the HKαα derivative and its reciprocal αααanti-4.2 derivative (see Fig. 4 in the online Data Supplement). The second involves unequal crossover between the X1 box of a wild-type allele and the X2 box of a −α3.7 allele, giving rise to the novel HKαα derivative and its reciprocal −α4.2 derivative (see Fig. 5 in the online Data Supplement). This is a likely mechanism of origin of the HKαα allele involving an intermediate allele, because the −α3.7 carrier state is quite common in the population. The third involves unequal crossover between a −α3.7 allele and an αααanti-4.2 allele, occurring in the X2 to Z2 region, leading to the HKαα derivative and a reciprocal αα (wild-type) derivative (see Fig. 6 in the online Data Supplement). This is a low-probability mechanism, given the rarity of concurrence of the −α3.7 and αααanti-4.2 alleles.

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References
7. Ma ESK, Chan AYY, Ha SY, Chan GCF, Au WY, Chan LC. The (−(SEA)) α-thalassemia (SEA) deletion ameliorates the clinical phenotype of β(0)/β(+) but not necessarily β(0)/β(0) thalassemia. Haematologica 2002;87:43–4.

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Expression of COX-2 mRNA in Peripheral Blood Monocytes from Patients with Acute Myocardial Infarction and Its Significance, Shui-ping Zhao,1 Ping Deng,2* Hong-guang Huang,2 Zhu-mei Xu,1 Hai-qing Dai,2 Shao-cai Hong,1 Jun Yang,1 and Hong-nian Zhou1 (1 Department of Cardiology, the Second XiangYa Hospital, Central South University, Hunan, China; 2 Changsha Central Hospital, Changsha, Hunan, China; * address correspondence to this author at: Department of Cardiology, Changsha Central Hospital, Shaoshan Road, No. 161, Changsha, Hunan 410014, China; fax 86-731-4895989, e-mail dengping2115@yahoo.com.cn)

Acute myocardial infarction (AMI) may occur when plaque ruptures and leads to thrombosis. Increasing evidence supports the notion that inflammation is involved in the atherogenesis and pathogenesis of AMI (1). Plasma concentrations of several markers of inflammation have been found to be associated with future cardiovascular risk in a variety of clinical settings. These markers include cell adhesion molecules, cytokines, proatherogenenic enzymes, and C-reactive protein (CRP). Interleukin-6 (IL-6), a central mediator of the acute-phase response (2), is positively correlated with the risk of future myocardial infarction (3). Matrix metalloproteinase-9 (MMP-9), an important member of the MMP family, may be responsible for the progress of extracellular matrix degradation in atherosclerotic plaques and plays an important role in plaque stability (4, 5). Its substrate types include collagen,
which is an essential component of the plaque basement membrane and fibrous cap. Systemic inflammatory markers such as IL-6 and MMP-9 are increased and become important proinflammatory cytokines in patients with AMI (1, 6–8).

Cyclooxygenase-2 (COX-2) is an inducible isofrom of COX, which is expressed in very limited fashion throughout most tissues unless induced by inflammatory stimuli or mitogens. COX-2 is the key enzyme that regulates the amount and duration of proinflammatory prostaglandins and plays an important role in inflammation (9, 10). It is now recognized that the antiinflammatory and analgesic actions of nonsteroidal antiinflammatory drugs are largely attributable to inhibition of COX-2 isoenzyme. COX-2 and prostaglandin E2 are known to induce production of the inflammatory cytokine IL-6 (11) and promote the release and activation of MMPs (12). Recent studies have shown that expression of COX-2 has also been specifically linked to cardiovascular disease. Because concentrations of the protein are increased in endothelial cells, smooth muscle cells, and macrophages in human atherosclerotic lesions (13–15), we hypothesized that COX-2 might have proinflammatory effects on patients with AMI.

Monocytes are one of the main cell types that express COX-2 (10). Monocyte/macrophage infiltration into the vessel wall is the key initial step in formation of atherosclerotic lesions. In this study, we measured COX-2 mRNA expression in peripheral blood monocytes from patients with AMI and investigated whether COX-2 expression is correlated to IL-6 and MMP-9 secretion by monocytes and whether a COX-2–specific inhibitor, celecoxib, can lower the secretion of IL-6 and MMP-9.

We recruited 40 patients with AMI (AMI group) and 18 individuals with stable coronary heart disease (CHD; control group) of similar age for the study. AMI was defined as a history of ischemic chest pain >30 min, characteristic electrocardiographic changes, and increased cardiac troponin I at least twice the upper limit of normal [as measured by ELISA (Biosseed, Inc.) with a cutoff value of 0.8 μg/L] within 24 h after the onset of pain. Stable CHD was defined as clearly established CHD or a positive exercise test with additional risk factors and no history of recent hospital admission (within the previous 3 months) for treatment of a coronary condition. None of the participants had hypertension, chronic heart failure, insulin-dependent diabetes mellitus, or inflammatory diseases (e.g., infections, malignancies, or autoimmune diseases). All participants had not received lipid-lowering drugs, antibiotics, salicylates, or glucocorticoids within 2 weeks. Peripheral blood monocytes were collected from all participants, and the monocytes from patients with AMI were collected within 12 h after the onset of pain. The study was approved by the local Institutional Review Board.

Peripheral venous blood from the patients with AMI (20 mL) or with stable CHD (5 mL) was layered on an equal volume of Ficoll Hypaque (Shanghai Biotech) and centrifuged at 1500g for 20 min at 25 °C. Peripheral blood mononuclear cells were harvested from the Ficoll-plasma interface and were washed twice with cold Hanks’ balanced salt solution (Sigma) and then once with serum-free RPMI 1640 (Gibco Life Technologies). The final pellet was suspended in serum-free RPMI 1640, and the cells were plated in 75-cm² flasks at a density 1 × 10⁶ cells/dish. Cell viability, as assessed by trypan blue exclusion, was routinely >95%. Nonadhering cells were removed after 6 h of incubation. Adhering monocytes were cultured for 24 h with RPMI 1640 supplemented with 2 mmol/L N-acetyl-l-alanyl-l-glutamine, 100 kilounits/L penicillin, 100 mg/L streptomycin, 20 g/L sodium pyruvate, 20 mmol/L HEPES (Gibco Life Technologies), and 100 mL/L heat-inactivated fetal calf serum (Gibco Life Technologies) at 37 °C in 5% CO₂. Cell culture supernatants were collected and stored at −70 °C for IL-6 and MMP-9 assays, and adhesive monocytes were used to extract total RNA.

For in vitro studies, peripheral blood mononuclear cells from patients with AMI were isolated as described above. After removal of the nonadhering cells, monocytes were exposed to various concentrations of celecoxib (0, 0.1, 1, and 10 μmol/L; product no. 169590-42-5; Hefei senrui) dissolved in dimethyl sulfoxide (Sigma) for 24 h, and culture supernatants were collected for IL-6 and MMP-9 assays.

Total RNA was extracted from monocytes by use of TRIzol reagent (Invitrogen Life Technologies, Gibco). RNA (1 μg) was converted to cDNA by standard techniques using a reverse transcription system (Promega). Amplification was performed at 94 °C (3 min) for denaturation followed by 38 cycles set of 50 s at 94 °C, 50 s at 55 °C, and 1 min at 72 °C with a final extension at 72 °C for 10 min. A primer pair specific to human COX-2 was synthesized according to sequences designed by Primer 3 software (sense, 5'-CAACCCACCGTGTTCTTCG-3'; antisense, 5'-CCATCCTTGAAAGGCGGAG-3'). The PCR product was 342 bp. Human cyclophilin (sense, 5'-ATGGTCAACCCCAAGCCTTTCTTCT-3'; antisense, 5'-CCATCCTTGAAAGGCGGAG-3'). The PCR product was 206 bp) was amplified as a reference for quantification of COX-2 mRNA. The reverse transcription-PCR product was separated and visualized by 1.5% agarose gel electrophoresis. The relative intensities of the bands of interest were measured on a Gel Doc2000 scanner (Bio-Rad) with scan analysis software and are reported as the ratio to the cyclophilin mRNA band.

Supernatant concentrations of IL-6 and MMP-9 were measured by specific sandwich ELISAs manufactured by Dalian Fangbang and R&D System. All samples were analyzed in duplicate. The detection limits were 1.0 ng/L and 2.0 μg/L, respectively, and the intra- and interassay CVs were both <10%.

All statistical analyses were carried out with SPSS 10.0 (SPSS Software). Numerical variables are presented as the mean (SD). Because some data were skewed, they were natural-log–transformed for all analyses. Comparisons between groups were analyzed by t-test (2-sided) or 1-way ANOVA followed by the Bonferroni test for experiments with more than 2 subgroups. Categorical variables...
were compared by χ² test. The association of measurements with other biochemical indices was assessed by the Spearman rank correlation test. We accepted statistical significance at P <0.05.

The clinical and biochemical characteristics of the participants are shown in Table 1. Sex distribution, age, body mass index, and blood pressure were not significantly different between patients with AMI and those with stable CHD. COX-2 expression in monocytes was analyzed by reverse transcription-PCR. We found that mean (SD) COX-2 expression was higher in peripheral blood monocytes from patients with AMI than in monocytes from controls [band intensity, 0.92 (0.13) vs 0.19 (0.08); Fig. 1A]. Compared with patients with stable CHD, peripheral blood monocytes from patients with AMI had increased IL-6 secretion [205 (46) vs 41 (8) ng/L; P <0.05] and MMP-9 secretion [192 (40) vs 22 (6) μg/L; P <0.05; Table 1]. Secretion of both IL-6 and MMP-9 by peripheral blood monocytes from patients with AMI was positively correlated with COX-2 mRNA expression in these cells (r = 0.636 and 0.853, respectively; P <0.05 for both).

Monocytes from patients with AMI were cultured for 24 h with celecoxib at concentration of 0, 0.1, 1, and 10 μmol/L, and the effects of celecoxib on secretion of IL-6 and MMP-9 were analyzed by ELISA. Celecoxib dramatically reduced the secretion of IL-6 and MMP-9 up to 48% and 50%, respectively (P <0.05 for both), in a concentration-dependent manner (Fig. 1B).

In our study, increased COX-2 expression was observed in peripheral blood monocytes from patients with AMI, and the correlation of COX-2 expression with IL-6 and MMP-9 secretion by monocytes was also strong. These results support the hypothesis of an acute inflammatory response to AMI that, at least in part, is correlated with COX-2 activation in peripheral blood monocytes. We also measured the secretion of IL-6 and MMP-9 by monocytes incubated with different concentrations of celecoxib and found that celecoxib dramatically reduced the in vitro secretion of IL-6 and MMP-9 in a concentration-dependent manner. These data suggest that COX-2 expression may promote secretion of IL-6 and MMP-9 by monocytes and that celecoxib might reduce acute atherosclerotic inflammation partly by inhibiting secretion of the inflammatory cytokines IL-6 and MMP-9 by peripheral blood monocytes. These findings are of particular clinical interest because CRP and MMP-9 are important markers of inflammation in AMI. IL-6 is a primary determinant of hepatic production of CRP (16), and higher CRP values indicate more severe AMI and a worsening prognosis (17, 18). The authors of recent experimental studies have reported that MMPs may potentially contribute to myocardial remodeling after AMI (19). As such, we believe our data support the possibility that COX-2–inhibiting therapy might provide a new approach to the acute inflammatory response to AMI.

Recent studies, both in vitro and in vivo, have suggested that COX-2 expression may promote atherosclerotic inflammation (13–15). COX-2–mediated eicosanoid production by activated macrophages may promote atherosclerosis through several mechanisms, including activation of chemotaxis, increased vascular permeability, propagation of the inflammatory cytokine cascade, stimulation of macrophage and smooth muscle cell migration, and stimulation of chemotaxis of human monocytes by LDL (9, 11, 12, 15, 20, 21). It has been reported that COX-2 is also correlated with endothelial dysfunction and oxidative stress, which promote the progress of atherosclerotic

| Table 1. Baseline characteristics of patients with AMI or stable CHD (controls) a,b,c |
|----------------------------------------|--------------|-----------------|---|
|                                      | Controls (n = 18) | AMI (n = 40) | P  |
| Age, years                           | 64.2 (9.5)     | 65.3 (10.1)    | NS |
| Men/Women, n                         | 14/4           | 30/10          | NS |
| BMI, kg/m²                            | 23.7 (5.1)     | 24.5 (4.1)     | NS |
| Smoking, n (%)                       | 12 (67%)       | 26 (65%)       | NS |
| SBP, mmHg                            | 125 (16)       | 127 (13)       | NS |
| DBP, mmHg                            | 75 (14)        | 76 (16)        | NS |
| WBC, x 10⁹/L                         | 6.8 (2.1)      | 7.1 (1.8)      | NS |
| Creatinine, μmol/L                   | 108.4 (18.2)   | 106.3 (9.6)    | NS |
| FBS, mmol/L                          | 5.26 (0.74)    | 5.52 (1.11)    | NS |
| IL-6, ng/L                           | 41 (8)         | 205 (46)       | <0.05 |
| MMP-9, μg/L                          | 22 (6)         | 192 (40)       | <0.05 |

a Values are the mean (SD) except for number of men and women and number (%) smokers.

b NS, not significant; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; WBC, leukocyte count; FBS, free blood sugar.

c P <0.05 vs control group.
inflammation. A clinical study has shown that COX-2–specific inhibition improves endothelial-dependent vasodilation and reduces low-grade chronic inflammation and oxidative stress in severe coronary artery disease (22). It has been observed that celecoxib produces a dose-dependent increase in nitrous oxide release and reduces the oxygen radical antioxidant capacity in primary cultures of human endothelial cells (23).

The Vioxx Gastrointestinal Outcomes Research (VIGOR) trial (24), however, showed that statistically more thromboembolic cardiovascular events occurred in individuals receiving a COX-2–specific inhibitor, rofecoxib. These observations have led to debates on the effects of COX-2 on cardiovascular events. By contrast, there was no increase in risk for patients receiving celecoxib when evaluated in either a prospective trial (25) or retrospective analyses (26–28). The basis for the observed differences in thrombosis risk among COX-2 inhibitors may be related to their distinct physicochemical properties (29). Rofecoxib is a sulfone with relatively poor tissue distribution, whereas celecoxib is a sulfonamide that has extensive tissue distribution. It has been shown that rofecoxib can cause a marked increase in nonenzymatic generation of isoprostanes and reduce the oxygen radical antioxidant capacity (23). One possible explanation for the results of VIGOR, therefore, may be that the increased risk for thromboembolic cardiovascular events is based on a nonenzymatic mechanism of rofecoxib and is not related with COX-2 inhibition.

References


Measurement of Pro-C-Type Natriuretic Peptide in Plasma

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Accurate measurement of peptides derived from pro-C-type natriuretic peptide (proCNP) in human plasma has been difficult for several reasons. Low plasma concentrations necessitate high assay sensitivity. In addition, suffi-