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The expression of monocyte chemotactic protein (MCP-1) in human vascular endothelium *in vitro* and *in vivo*

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Abstract

A monocyte chemotactic protein (MCP-1) is thought to play a major role in recruiting monocytes to the vascular endothelium where the adherence of monocytes is one of the earliest events in atherogenesis. We cloned MCP-1 cDNA from a λgt 11 cDNA library constructed from human aortic endothelial mRNA to test whether MCP-1 expressed in arterial endothelium is identical to those from other sources. A ~ 670 bp MCP-1 cDNA clone was identified and showed the identical sequence with the ones from other cell lines. Northern blot analysis using this cloned MCP-1 cDNA as probe revealed two hybridizing bands of RNA at 0.68 and 0.77 kb in human aortic, human pulmonary arterial, and human umbilical vein endothelial cell cultures. Primer extension analysis showed that the difference in size (~ 90 bp) between the two transcripts is not due to a difference at the 5′-noncoding region. The amount of MCP-1 transcripts increased dramatically in aortic endothelial cells when stimulated with recombinant IL-1α (100 units/ml), IL-1β (100 units/ml), or TNF-α (200 ng/ml). Northern blot and slot blot analysis of RNA isolated from both the endothelium and the underlying vessel wall of freshly removed human arteries and veins showed MCP-1 transcripts. This observation demonstrates for the first time that MCP-1 is expressed not only in atherosclerotic human arteries but also in symptom free arteries and veins *in vivo.* (Mol Cell Biochem **126:** 61–68, 1993)

Key words: MCP-1, endothelium, JE gene, atherosclerosis, cytokine

Introduction

The adherence of peripheral blood monocytes to the vascular endothelium is one of the earliest events that occurs in atherogenesis [1–3]. Monocyte chemoattractants released at the foci of endothelial injury are thought to play an important role in this cellular interaction [4–6]. A monocyte chemotactic protein (MCP-1) has been purified from various tissue sources. MCP-1

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obtained from baboon smooth muscle cells was found to be a monomeric protein with a molecular weight of 14,500 [7], whereas the chemotactic proteins generated by malignant cells contained two immunoreactive MCP-1 like proteins with MW. of 9,000 and 14,000 [8]. Similar proteins were also purified from human glioma and leukocyte cell lines [9, 10]. MCP-1 seems to be important not only for macrophage infiltration of tumor tissue and for host defense in local inflammation, but may also play a critical role in recruiting monocytes to stressed endothelium as a response to vascular injury. For example, minimally modified LDL (MM-LDL) has been reported to activate the expression of MCP-1 in cultured endothelial cells and in mice models *in vivo* [11, 12]; transmigration of monocytes induced by LDL into the subendothelial space was inhibited by antibody to MCP-1 [13]. Very little is known about MCP-1 production by vascular tissues *in vivo*. Recently MCP-1 production was detected in the regions of arterial tissues that show clinical symptoms of atherosclerosis [27]. The cDNA for MCP-1 from glioma cells and promyelocytic cells was recently cloned and the nucleotide sequence was determined [14, 15]. A single 0.7 Kb mRNA species was detected as the MCP-1 gene product in human fibroblasts and endothelium [16, 17].

In this paper, we report that the sequence of MCP-1 cDNA cloned from human aortic endothelial cells is identical to the ones from other cell lines. Northern blot analysis revealed the presence of two MCP-1 transcripts in human vascular endothelial cells, whereas only a single transcript has been reported by others. We demonstrate that MCP-1 gene transcripts are present not only in human arterial regions that show plaques but also in regions of the artery of older patients that do not show any visible plaques of any kind. For the first time, it is also shown that veins can also express the MCP-1 gene.

Materials and methods

Cell culture

Human umbilical cords were obtained from The Ohio State University Hospitals. Human umbilical vein endothelial cells (HUVE) were harvested using published procedures [18, 19]. Human aortic and pulmonary endothelial cells were kindly provided by Dr. A.S. Antonov of the Cardiovascular Research Institute of the USSR. Cells were
ultured to confluence in M-199 medium supplemented with 10% fetal bovine serum and endothelial cell growth factor in an atmosphere of 5% CO_2 at 37° C. All the cells used for experiments were prior to seventh passage. In cytokine stimulation experiments, the confluent monolayer was washed twice with phosphate-buffered saline (PBS) and cultured in media supplemented with recombinant human interleukin-1α (IL-1α, 100 units/ml), interleukin-1β (IL-1β, 100 units/ml), tumor necrosis factor (TNF-α, 200 ng/ml), interleukin-2 (IL-2, 100 units/ml), interleukin-6 (IL-6, 200 units/ml), or interferon-γ (INF-γ, 200 units/ml). After incubation for 1 hr, the media was removed and the monolayer was washed with PBS three times before RNA extraction. All the cytokines were obtained from Boehringer Mannhein (Indianapolis, IN).

DNA library construction and screening

Double-stranded cDNA was prepared from the poly $(A)^+$ RNA from human aortic endothelial cells using the cDNA synthesis kit (Pharmacia). After ligation with *EcoRI* linker, the cDNA was cloned into the *EcoRI* site of λ*gt*11 vector. A cDNA library of 10^6 recombinants was obtained and screened with a 52-mer oligonucleotide probe labeled with ^{32}P at the 5′-end. This synthetic probe, having sequence ATCACAGCTTCTTTGG-GACACTTGCTGCTGGTGATTCTTCTATAGC-TCGCGA was based on human glioma MCP-1 cDNA [14, 15]. Positive clones were selected and purified after repeated screening to achieve uniformly positive hybridization. DNA was isolated from the positive clones and subcloned into Bluescript cloning vector. DNA sequencing was carried out by the dideoxynucleotide method of Sanger *et al.* [20].

Northern and slot blot analysis

Total cellular RNA was isolated from cultured endothelium using the guanidine isothiocyanate method [21]. In brief, the monolayer was lysed in 4 M guanidine isothiocyanate with 0.5% sarkosyl. The cell extract was layered on a cushion of 5.7 M CsCl and ultracentrifuged at 42,000 rpm for 18 hr. The RNA sediment on the bottom of the ultracentrifuge tube was recovered and purified by ethanol precipitation. Poly$(A)^+$ RNA was subsequently isolated by oligo(dT) cellulose column chromatography.

Northern blot analysis was performed according to the procedure described by Fourney *et al.* [22]. Either

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                                TCAAACTGAAGCTCGCACTCTCGCCTCCAGC
  +1 ATG AAA GTC TCT GCC GCC CTT CTG TGC CTG CTG CTC ATA
     Met Lys Val Ser Ala Ala Leu Leu Cys Leu Leu Leu Ile

     GCA GCC ACC TTC ATT CCC CAA GGG CTC GCT CAG CCA GAT
     Ala Ala Thr Phe Ile Pro Gln Gly Leu Ala Gln Pro Asp

 +79 GCA ATC AAT GCC CCA GTC ACC TGC TGC TAT AAC TTC ACC
     Ala Ile Asn Ala Pro Val Thr Cys Cys Tyr Asn Phe Thr

     AAT AGG AAG ATC TCA GTG CAG AGG CTC GCG AGC TAT AGA
     Asn Arg Lys Ile Ser Val Gln Arg Leu Ala Ser Tyr Arg

+157 AGA ATC ACC AGC AGC AAG TGT CCC AAA GAA GCT GTG ATC
     Arg Ile Thr Ser Ser Lys Cys Pro Lys Glu Ala Val Ile

     TTC AAG ACC ATT GTG GCC AAG GAG ATC TGT GCT GAC CCC
     Phe Lys Thr Ile Val Ala Lys Glu Ile Cys Ala Asp Pro

+235 AAG CAG AAG TGG GTT CAG GAT TCC ATG GAC CAC CTG GAC
     Lys Gln Lys Trp Val Gln Asp Ser Met Asp His Leu Asp

     AAG CAA ACC CAA ACT CCG AAG ACT TGA ACACTCACTCCACAA
     Lys Gln Thr Gln Thr Pro Lys Thr

+316 CCCAAGAATCTGCAGCTAACTTATTTTCCCCTAGCTTTCCCCAGACATCCT
     GTTTTATTTTATTATAATGAATTTTGTTTGTTGATGTGAAACATTATGCCT
+418 TAAGTAATGTTAATTCTTATTTAAGTTATTGATGTTTTAAGTTTATCTTTC
     ATGGTACTAGTGTTTTTTAGATACAGAGACTTGGGGAAATTGCTTTTCCTC
+520 TTGAACCACAGTTCTACCCCTGGGATGTTTTGAGGGTCTTTGCAAGAATCA
     TTTTTTTAACATTCCAATGCATTTAATACAAAGAATTGCTAAAATATTATT
+622 GTGGAAATG
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Fig. 1. MCP-1 cDNA sequence from human aortic endothelial cells. The C at position + 105 (underlined) is the one which shows polymorphism of the cDNA sequence from other cell lines. This change does not alter the Cys amino acid residue.

20 μg of total cellular RNA or 5 μg of poly(A)$^+$ RNA was electrophoresed through a 1.7% agarose-formaldehyde gel, followed by transfer to a nylon membrane. The filter was pre-hybridized in 50% (v/v) formamide, 6 × SSC, 0.1% (v/v) SDS, 5 × Denhardt's solution, and 100 μg/ml salmon sperm DNA for 4 hr, and then hybridized at 42° C for 24 hr with a ^{32}P-labeled cDNA or a 52-mer oligonucleotide probe complementary to MCP-1 mRNA. After hybridization, the nitro-cellulose membrane was washed and placed against Kodak XAR film for autoradiography. Slot blot was performed in a similar manner as Northern blot analysis except that 1 μg of Poly(A)$^+$ RNA was directly loaded onto the filter.

Primer extension analysis

The synthetic 52-mer oligonucleotide which is located at position + 143 to + 194 of the cDNA was used as an extension reaction primer and was labeled with ^{32}P at the 5′-end. Two micrograms of poly(A)$^+$ mRNA or 10 μg of cellular RNA isolated from cultured human umbilical vein endothelial cells treated with 15 μg/ml bacterial lipopolysaccharides (LPS) were mixed with 2×10^4 cpm of the labeled primer. After denaturing by heat treatment, the mixture was annealed overnight. The primer was extended to the 5′-end by the addition of 200 units MMLV reverse transcriptase. The reaction products were analyzed on 5% acrylamide/8 M urea sequencing gel. Size was determined by a DNA sequencing ladder run in parallel lanes.

Examination of human vessel tissues for MCP-1 gene transcripts

The vessel tissues, arteries or veins, were surgically removed from patients and within minutes placed into liquid nitrogen and stored at − 70° C until the time for RNA extraction. Cellular RNA was isolated from tis-

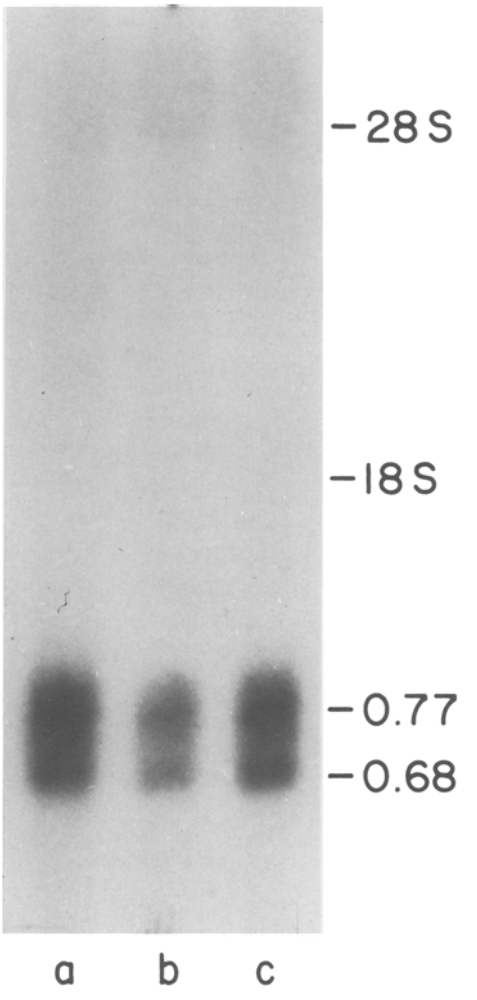

Fig. 2. Northern blot analysis of MCP-1 gene transcripts in human aortic (a), pulmonary arterial (b) and umbilical venous (c) endothelial cell cultures. Cells were cultured for two days without changing culture media, the total RNA (20 μg each) was subjected to electrophoresis and the blots were probed with ^{32}P-labeled MCP-1 cDNA. The location of ribosomal RNA bands and the size of the MCP-1 mRNA bands are shown on the right side.

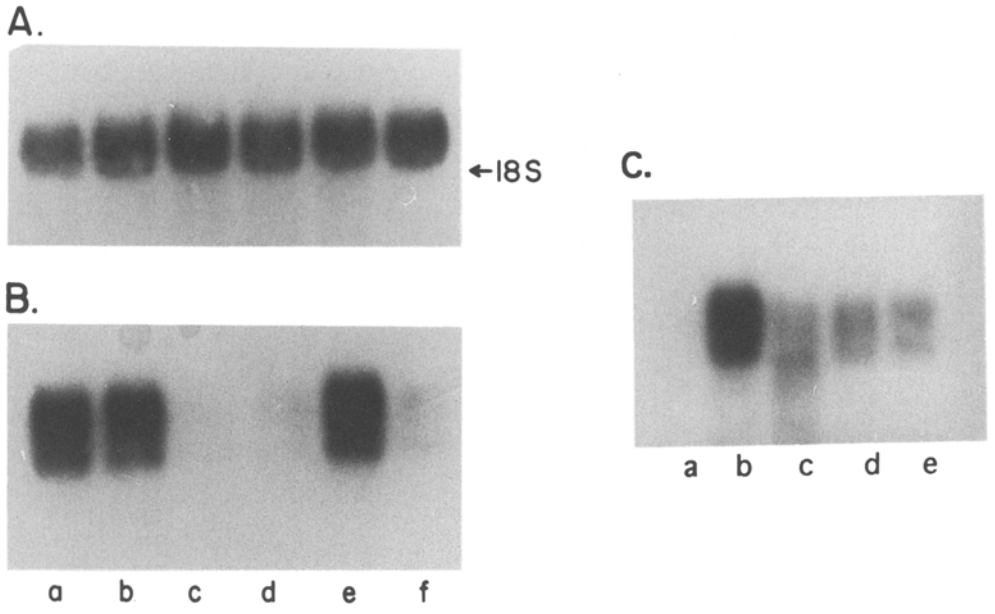

Fig. 3. Effects of cytokines on the level of MCP-1 gene transcript in human endothelial cells. Total RNA was isolated from HUVE cells which were treated with (a) 100 units/ml IL-1α, (b) 100 units/ml IL-1β, (c) 100 units/ml IL-2, (d) 200 units/ml IL-6, (e) 200 ng/ml TNF-α and (f) 200 units/ml INF-γ. 20 μg total RNA of each treatments was subjected to electrophoresis and probed with β-actin as internal control (A) and MCP-1 cDNA probe (B). RNA also isolated (20 μg) from human aortic endothelial cells without treatment (a), or treated with 15 μg/ml LPS (b), 200 ng/ml TNF-α (c), 100 units/ml IL-1α (d), and 100 units/ml IL-1β (e) were subjected to northern blot analysis. The transcripts were hybridized with ^{32}P-labeled MCP-1 cDNA probe (C).

sues as previous described [23]. In brief, the frozen tissues were ground into fine powder which were then dissolved in 10 volume of guanidine isothiocyanate lysis buffer (4 M guanidine isothiocyanate, 5 mM sodium citrate, 0.5% sarkosyl, and 0.1 M β-mercaptoethanol). The tissue mixture was further homogenized to a slurry with a Polytron and cellular RNA was precipitated with ethanol. The pellet was dissolved in a small volume of lysis buffer and applied to CsCl gradient for ultracentrifugation as described above.

Results

Isolation of cDNA clones and nucleotide sequencing

A λ*gt* 11 cDNA library prepared from poly(A)$^+$ RNA isolated from cultured human aortic endothelial cells was screened with a 52-mer oligonucleotide probe. Screening 10^6 recombinants yielded 30 positive cDNA clones. The complete nucleotide sequences of the insert portions of cDNA were determined. The sequence contains a 5′-noncoding region of 31 bp, a 297 bp open reading frame starting with ATG, and a 3′-noncoding region of 331 bp. In the entire sequence, only one residue, C at +105, mismatched with T in glioma MCP-1 cDNA sequence. This change does not alter the amino acid sequence (Fig. 1).

The expression of MCP-1 gene in cultured vascular endothelial cells

RNA prepared from human aortic endothelial, human umbilical venous endothelial, and human pulmonary arterial endothelial cells were subjected to Northern blot analysis using the isolated cDNA fragment as a probe. Two bands of RNA at 0.68 and 0.77 Kb in all three types of endothelial cell cultures cross-hybridized with MCP-1 cDNA probe (Fig. 2). To test the effect of LPS and cytokines in up-regulating MCP-1 gene expression in endothelium, confluent HUVE cells were treated with IL-1α (100 units/ml), IL-1β (100 units/ml), IL-2 (100 units/ml), IL-6 (200 units/ml), TNF-α (200 ng/ml) and IFN-γ (200 units/ml) for 7 hr followed by RNA extraction. Northern blot analysis (Fig. 3) shows that the levels of MCP-1 transcripts can be elevated by IL-1α, IL-1β and TNF-α. Similar experiments were performed on human aortic endothelial cells. The results are shown in Fig. 3C.

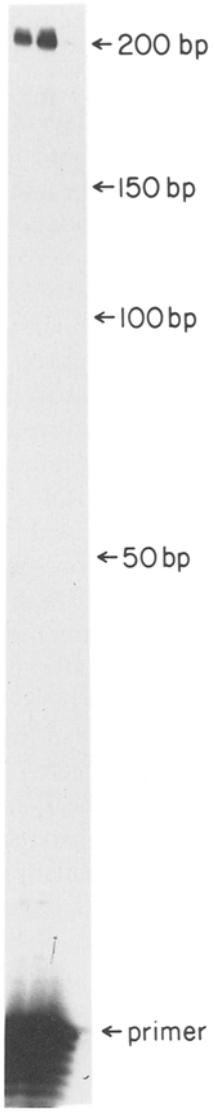

Fig. 4. Primer extension analysis of the transcription starting site for the human MCP-1 gene. A ^{32}P-labeled 52-mer oligonucleotide complementary to MCP-1 message (+ 143 to + 194) was annealed to mRNA isolated from LPS-treated human umbilical vein endothelial (left lane) and human aortic endothelial (right lane) cell cultures. The primer was extended to the 5′-end by MMLV reverse transcriptase and the products were analyzed on a sequencing gel.

Primer extension analysis

To determine whether the difference in size between the two MCP-1 transcripts is due to a difference at the 5′-noncoding region, primer extension analysis was carried out. The 52-mer oligonucleotide complementary to MCP-1 message (+ 143 to + 194) was employed as primer and annealed to mRNA isolated from LPS-treated human aortic endothelial cells. As shown in Fig. 4, a single product composed of 200 bases was observed. This size corresponds to the presence of 57 bases at the 5′-flanking region of the mRNA. The single product obtained in this experiment indicates that the 5′-noncoding regions of the two transcripts observed in the northern blot are identical in size.

Expression of MCP-1 gene in human vascular tissues

MCP-1 gene expression in vascular tissue *in vivo* is demonstrated by northern blot and slot blot analysis (Fig. 5). RNA isolated from surgically removed arteries of several patients hybridized with the MCP-1 cDNA probe. It is noteworthy that the MCP-1 transcript levels found in segments of arteries from the region of the atherosclerotic plaque were not always higher than the levels

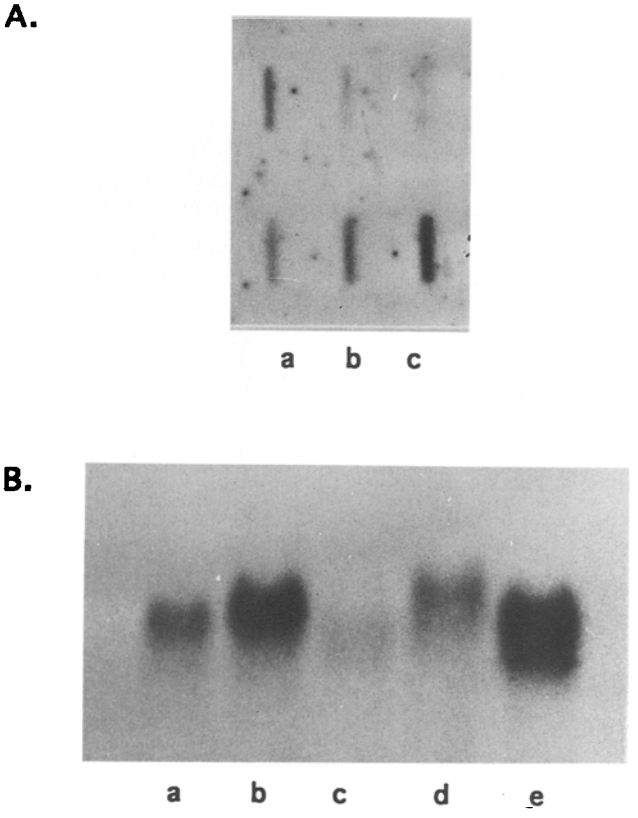

Fig. 5. MCP-1 gene expression in surgically removed human vascular tissues. (A) Slot blot analysis of RNA isolated from abnormal artery (a), normal artery (b), and vein (c) of a 72 year old female (top) and 78 year old diabetic male (bottom). (B) Northern blot analysis of RNA isolated from the aorta of a 54 year old male with lesion stage 4 (a), the aorta of a 65 year old male patient with lesion stage 2 (b), the femoral artery of a 78 year old male with lesion stage 3 (c), the iliac artery of a 31 year old female with lesion stage 4 (d), (e) shows the iliac artery adjacent to (d) with lesion stage 1. The probe was ^{32}P-labeled MCP-1 cDNA. Each blot presents 5 μg RNA in (A) and 20 μg RNA in (B).

found in adjacent segments that did not have visible lesions, although in some cases MCP-1 transcripts were only found in the lesion area. Arterial segment with advanced, highly calcified lesions showed lower amount of MCP-1 transcripts.

We also tested whether MCP-1 transcripts are found in human venous tissues *in vivo*. Hybridization of RNA from artery and vein samples from two patients showed that the vein of one diabetic patient had as much or more MCP-1 transcripts as found in the arterial segment from the same patient, whereas only the artery but not the vein from the other patient showed MCP-1 transcripts (Fig. 5B). Northern blot analysis of RNA from an 87-year old female also showed the two MCP-1 transcripts in both the artery and vein samples whereas an 86-year old female showed the MCP-1 transcripts only in the arterial sample (data not shown).

Discussion

Atherogenesis involves the initial attraction and attachment of peripheral blood monocytes to the endothelial cells, the migration of the monocytes into the subendothelial space, and their differentiation into macrophages with subsequent uptake of lipids to become foam cells [1–3]. This process probably involves recruitment of monocytes by endothelial cells that have been stimulated to release the monocyte chemotactic factor by risk factors such as stress stimuli, oxidized LDL and injury. The monocytes thus recruited adhere to vascular endothelium and initiate atherogenesis. MCP-1 may significantly contribute to the endothelial recruitment of monocytes. The nucleotide sequence of the MCP-1 cDNA from glioma cells and promyelocytic cell lines have been deduced previously [14, 15]. We tested whether the MCP-1 from human aortic endothelial cells was identical to those from other tissue sources by cloning MCP-1 cDNA from a human aortic endothelial cDNA library with a 52-mer oligonucleotide synthesized on the basis of glioma MCP-1 cDNA sequence. The nucleotide sequence of the clone sequenced shows that the open reading frame of 297 bp was identical to that of glioma cell MCP-1 except for a C that replaced T at position 105 [15]. However, this single base change does not alter the amino acid sequence and therefore the cloned cDNA was used as the probe for MCP-1 gene expression analysis.

Our Northern blots showed two species of MCP-1 gene transcripts, whereas a single class of MCP-1 mRNA with the size of 0.7 Kb was reported in human umbilical vein endothelial cells, human fibroblasts, and human glioma cell lines [14, 16, 17]. Since the ribosomal RNA bands showed no sign of degradation and the probing for β-actin mRNA also showed one sharp band, the two MCP-1 bands we observed could not have been the result of general RNA degradation. To test whether the two transcripts originated by the use of two transcription start sites, a primer extension analysis was done with a 52-mer oligonucleotide that spans + 143 to + 194 of the cDNA. The single band obtained showed that the 5′-flanking region contained 57 bases which is similar to the size of the 5′ region observed in human glioma and promyelocytic cells. Since only one product was obtained from the poly(A)$^+$ RNA, it seems unlikely that the two mRNA species have differences at the 5′ end. This conclusion is based on the assumption that the primer could hybridize with both mRNA species. If the amounts of primer extension products generated from the two species of mRNA reflected the relative amounts indicated in the northern blots, the primer extension analysis would have revealed two products. Thus, the two species of mRNA detected probably differ at the 3′-end. Polymorphism in the 3′-end of mRNA has been observed in several gene products including granulocyte-monocyte colony stimulating factor (GM-CSF) in T lymphocytes [24], and c-*myc* and c-*fos* in NIH 3T3 cells [25, 26]. Published northern blots of MCP-1 transcripts from Mark's report [17] also show indications of a double band although the authors did not recognize the possibility of two mRNA species. A common sequence AUUUA in the 3′-untranslated region of these mRNA was suggested to be the site of cleavage. We also found such an AU-rich sequence located at + 436 and + 440 in the 3′-untranslated region of the cloned MCP-1 cDNA. Whether the shorter mRNA species is the cleavage product of the longer one and the occurrence of the two species has any functional significance need further investigation.

MCP-1 gene expression can be viewed as a response to stress. In the absence of a stimulant, MCP-1 transcripts were detected in all three types of vascular endothelial cells after two days in culture without changing medium. Presumably, the stress caused by the nutritional deficiency resulting from the long incubation or accumulation of some metabolites triggered the expression of this gene. Consistent with our observation is the report that MCP-1 was isolated from the conditioned media of baboon vascular smooth muscle cells cultured in the absence of serum for two days [7]. Even transfection pro-

cedures using lipofectin that presumably caused membrane perturbation also triggered the expression of endogenous MCP-1 gene (unpublished results). Furthermore, IL-1α, IL-1β, TNF-α, and LPS that are known to be signals that indicate stress such as injury and infection, all triggered MCP-1 gene expression in arterial endothelium.

While the present work was in progress it was reported that expression of MCP-1 could be detected in macrophage-rich regions of human and rabbit atherosclerotic lesions by *in situ* hybridization and immunocytochemical methods [27]. Our demonstration of the presence of MCP-1 transcripts identified by northern blots agree with this conclusion. In addition, we find that MCP-1 gene transcript is found not only in the freshly harvested human artery but also in veins. Since these tissues were frozen within minutes after excision from the donors, it is highly unlikely that MCP-1 mRNA we observed were synthesized in the tissue after excision as a result of perception of some injury signal nor was it due to a general degradation as indicated by the sharp band that hybridized to the MCP-1 probe. The different levels of expression of MCP-1 in human aortic tissue with different stages of lesions imply that this gene may play an important role in atherogenesis. Released from early stage of the stressed or injured endothelium, MCP-1 may function as a signal to attract blood monocytes to the site of injury. Our results show that the level of MCP-1 does not directly correlate with the severity of the lesions. Even normal looking segments of the blood vessel could have certain higher levels of the transcript than the levels found in lesions at advanced stages. Even though MCP-1 was not previously detected in veins, we found that in some patients the venous tissues do express MCP-1 at fairly high levels. From the admittedly limited number of patients we examined, it appears that under pathological conditions of advanced involvement of atherosclerosis, both the arteries and veins produce MCP-1.

Additionally, the differentiated marcrophage in the vascular wall may release regulatory molecules such as IL-1α, IL-1β, and TNF-α. These cytokines would increase the expression of MCP-1 from surrounding tissue (e.g., vascular endothelium and smooth muscle cells). As a result of a localized MCP-1 concentration gradient, additional monocytes would be attracted from the circulating blood. This gradient may prevent trapped monocytes in the vascular wall from reentering the circulating blood, which cause monocyte accumulation in the subendothelial space. Such a process may promote the differentiation of monocytes to macrophages with subsequent LDL deposition and development into foam cells.

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