Human Alveolar Macrophages Are Markedly Deficient in REF-1 and AP-1 DNA Binding Activity*

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Although many functions of human alveolar macrophages are altered compared with their precursor cell, the blood monocyte (monocyte), the reason(s) for these functional changes have not been determined. We recently reported that human alveolar macrophages do not express AP-1 DNA binding activity (Monick, M. M., Carter, A. B., Gudmundsson, G., Geist, L. J., and Hunninghake, G. W. (1998) Am. J. Physiol. 275, L389—L397). To determine why alveolar macrophages do not express AP-1 DNA binding activity, we first showed that there was not a decrease in expression of the FOS and JUN proteins that make up the AP-1 complex. There was, however, a significant difference in the amounts of the nuclear protein, REF-1 (which regulates AP-1 DNA binding by altering the redox status of FOS and JUN proteins), in alveolar macrophages compared with monocytes. In addition, in vitro differentiation of monocytes to a macrophage-like cell resulted in decreased amounts of REF-1. Finally, addition of REF-1 from activated monocytes to alveolar macrophage nuclear proteins resulted in a marked increase in AP-1 DNA binding. These studies strongly suggest that the process of differentiation of monocytes into alveolar macrophages is associated with a loss of REF-1 and AP-1 activity. This observation may explain, in part, some of the functional differences observed for alveolar macrophages compared with monocytes.

Alveolar macrophages are critical cells that are important for pulmonary host defense and the development of inflammation in the lung (1—12). Alveolar macrophages are derived from monocytes after migration of the cells into the lung and differentiation into more mature macrophages (13, 14). During the process of differentiation, there are a number of phenotypic changes that result in an increased capacity to adhere to various surfaces, an increased phagocytic ability, a difference in morphology, and a changed ability to secrete cytokines (15, 16).

Although these differences in phenotype are well described, there is little information about changes in signal transduction pathways that might mediate the different functions of these two cell types. The only relevant studies are those of Peters-Golden et al. (14) and a study by Monick et al. (11). Both of these studies noted that normal alveolar macrophages had a decreased capacity to express protein kinase C activity, compared with monocytes or other macrophages. In the study by Monick et al. (11), we also found that normal human alveolar macrophages had a decreased capacity to express protein kinase C-induced AP-1 DNA binding.

There are a number of FOS- and JUN-like proteins that can form the heterodimers or homodimers that make up the AP-1 complexes (17—20). The classic complex is comprised of c-FOS and c-JUN, and these proteins are found in the nucleus of cells. AP-1 activity can be regulated at many levels, including transcription of genes that code for AP-1 proteins, message stability, and translation of the mRNAs. AP-1 activity is also regulated by the composition of the AP-1 complexes, phosphorylation of the proteins, and redox regulation of cysteine residues in the AP-1 proteins (17—21). The redox status of the AP-1 protein complex determines the binding of AP-1 to DNA. A dual function nuclear protein called REF-1 (or apurinic/apyrimidinic endonuclease DNA repair enzyme) regulates the binding activity of AP-1 (22—25). This protein has two distinct and separate functions that involve different parts of the protein. REF-1 repairs apurinic/apyrimidinic sites in DNA, and it is activated by thioredoxin to reduce cysteine residues on both FOS and JUN enabling DNA binding by AP-1 (22—25).

This study analyzed possible mechanisms for the lack of AP-1 DNA binding in alveolar macrophages. We found no differences in expression of c-FOS or c-JUN proteins in alveolar macrophages compared with monocytes at base line or after protein kinase C stimulation. We did find that alveolar macrophages are deficient in REF-1. Reconstituting alveolar macrophage nuclear proteins with monocyte-derived REF-1 reconstituted AP-1 binding activity. In addition, when monocytes were allowed to differentiate into macrophages, in vitro, they lost REF-1 and the ability to respond to PMA1 with increased AP-1 binding. We conclude that the process of differentiation of monocytes into alveolar macrophages is accompanied by a loss of REF-1, affecting AP-1 binding and subsequent expression of AP-1-driven genes.

MATERIALS AND METHODS

Isolation of Human Alveolar Macrophages—Alveolar macrophages were obtained from bronchoalveolar lavage as described previously (5). Briefly, normal volunteers with a lifetime non-smoking history, no acute or chronic illness, and no current medications, underwent bronchoalveolar lavage. The lavage fluid was filtered through two layers of gauze and centrifuged at 1500 × g for 5 min. The cell pellet was washed twice in Hanks’ balanced salt solution without Ca++ and Mg++ and suspended in complete medium, Roswell Park Memorial Institute (RPMI) tissue culture medium (Life Technologies, Inc.), with 5% fetal calf serum (HyClone, Logan, UT) and added gentamycin (80 µg/ml). Differential cell counts were determined using a Wright-Giemsa-stained cytocentrifuge preparation. All cell preparations had between 90 and 100% alveolar macrophages. This

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1 The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; RPMI, Roswell Park Memorial Institute; LPS, lipopolysaccharide.
study was approved by the Committee for Investigations Involving Human Subjects at the University of Iowa.

Isolation of Human Blood Monocytes—180 ml of heparinized blood was obtained by venipuncture of the same volunteers who underwent bronchoscopy. Monocytes were isolated using a Ficoll-Paque gradient (S人际ton). After harvesting the mononuclear cell layer, cells were washed four times in phosphate-buffered saline and then resuspended in RPMI medium. Additional purification was obtained by a 1-h adherence at 37 °C. Non-adherent cells were then washed off, and RPMI medium was added back to the adherent cells. In some instances the monocytes were allowed to differentiate into more macrophage-like cells. In order to do this, adherent monocytes were cultured in RPMI with 100 ng/ml human AB serum for a period of 7–10 days. At the end of that period, floating cells were washed off, and the remaining adherent cells had spread out and obtained the pancake-like appearance of macrophages.

Isolation of Nuclear Extracts and Electrophoretic Mobility Shift Assays—Alveolar macrophages and monocytes were cultured for 3 h with or without 100 ng/ml PMA. The nuclear pellets were prepared by resuspending cells in 0.4 ml of lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA), placing them on ice for 15 min, and then vigorously mixing after the addition of 25 μl of 10% Nonidet-40. After a 30-s centrifugation (16,000 × g, 4 °C), the pelleted nuclei were resuspended in 50 μl of extraction buffer (50 mM HEPES, pH 7.8, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol) and incubated on ice for 30 min. Nuclear extracts were stored at 70 °C. The DNA binding reaction (electrophoretic mobility shift assay) was done at room temperature in a mixture containing 5 μg of nuclear proteins, 1 μg of poly(dI-dC), and 15,000 cpm of 32P-labeled double-stranded oligonucleotide probe for 30 min. The samples were fractionated through a 5% polyacrylamide gel in 1× TBE (Tris base 6.05 g/liter, boric acid 3.06 g/liter, EDTA-Na₂ 0.37 g/liter). Sequence of the nucleotide was 5’-CCGGTTGTAGTCGCCGGA-3’ (AP-1). Experiments were repeated 3 times. Supershift assays were performed by incubating the nuclear protein with antibodies specific for c-FOS and c-JUN (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at room temperature before the addition of the labeled DNA probe. Specific c-FOS and c-JUN proteins are indicated by a higher band on the gel and by an overall decrease in binding.

Isolation of RNA and Northern Analysis—Whole-cell RNA was isolated using RNA Stat-60 according to the manufacturer’s instructions (Tel-Test “B”, Friendswood, TX). The isolated RNA was fractionated in a 1.5% denaturing agarose gel containing 2.2 M formaldehyde. An RNA ladder (0.25–9.5 kilobase pairs; Life Technologies, Inc.) was included as a molecular size standard. RNA loading was confirmed by equivalent ethidium bromide staining in each lane. The RNAs were transferred to GeneScreen Plus (NEN Life Science Products) as suggested by the manufacturer. c-FOS and c-JUN cDNA probes (generated by polymerase chain reaction with primers obtained from CLONTECH, Palo Alto, CA) were labeled with γ-32P[dCTP (NEN Life Science Products) by the random primer method. Blots were prehybridized for 3 h at 42 °C (formamide 10 ml, NaCl 5 mm, 50% dextran 4 ml, 10% SDS, Tris, pH 7.0, 1x, and 0.4 ml 50× Denhardt’s solution) and then hybridized with the labeled probe overnight at 42 °C. The filters were washed twice with 1× SSC at 25 °C, twice with 1× SSC plus 1% SDS at 65 °C, and then once with 0.1× SSC at 25 °C. The filters were exposed to autoradiographic film at −70 °C.

Western Analysis—For these studies, alveolar macrophages and monocytes were cultured for 3 h with or without PMA (10 or 100 ng/ml). At the end of the culture period, either whole-cell protein extracts or nuclear protein extracts (see gel shift protocol) were obtained as described previously (11). The cell material was sonicated 15 s on ice, allowed to sit for 20 min, and then centrifuged at 15,000 × g for 10 min. An aliquot of the supernatant was used to determine protein concentration by the Coomassie Blue method. Equal amounts of protein (100 μg for whole cell extracts and 20 μg for nuclear extracts) were mixed 1:1 with 2× sample buffer and loaded onto a 10% SDS-polyacrylamide gel and run at 80 V for 2 h. Cell proteins were transferred to nitrocellulose (ECL, Amersham Pharmacia Biotech) overnight at 30 V and visualized using c-FOS, c-JUN, or REF-1-specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were developed using a chemiluminescent substrate (ECL, Amersham Pharmacia Biotech).

RESULTS

PMA Induces Increased AP-1 DNA Binding in Monocytes but Not Alveolar Macrophages—In order to evaluate the effect of PMA (a model of protein kinase C-driven activation) on AP-1 DNA binding in monocytes and alveolar macrophages from the same individual, cells were cultured for 3 h with and without PMA (10 ng/ml) and LPS (1 μg/ml) for 3 h. Nuclear proteins were isolated, and DNA binding was evaluated in a standard gel shift assay. In Fig. 1A, we show that PMA caused an increase in protein binding to the AP-1 consensus sequence with nuclear protein from monocytes but not with nuclear protein from alveolar macrophages. LPS did not increase AP-1 binding in monocytes or alveolar macrophages. In Fig. 1B, we use cold competition to show that it is the upper band (seen only with monocyte samples) that can be competed off with cold AP-1 oligonucleotide. In order to confirm that the alveolar macrophages were functional, we also evaluated binding to the NF-κB sequence. We showed that LPS increases both monocyte and alveolar macrophage nuclear protein binding to the NF-κB consensus sequence (data not shown).

In Fig. 2, we show that PMA causes an increase in AP-1 binding in monocytes over a prolonged time course (0.5–6 h). At none of these time points was there the appearance of AP-1 binding activity in alveolar macrophages. These data show that the difference in AP-1 binding activity between monocytes and macrophages is not simply a function of different response times.

PMA Induces c-FOS and c-JUN mRNA and Protein in Both Monocytes and Alveolar Macrophages—One possible explanation for the lack of AP-1 DNA binding in alveolar macrophages is that alveolar macrophages do not make the same amounts of AP-1 proteins as monocytes. We initially evaluated the composition of the PMA-induced AP-1-binding proteins in monocytes with a supershift assay. Both the upward shift of the band and the decrease in binding shown in Fig. 3 demonstrate that the monocyte AP-1 complex includes both c-FOS and c-JUN proteins. We next analyzed both cell types for the production of specific c-FOS and c-JUN mRNAs. Cells were cultured for 3 h, as in the gel shift experiments, and then harvested- and whole-cell RNA was extracted (Fig. 4A). Both monocytes and alveolar macrophages responded to PMA with increased amounts of c-FOS and c-JUN message. Compared with monocytes, the alveolar macrophages showed increased amounts of both c-FOS and c-JUN mRNA. We next evaluated c-FOS and c-JUN pro-
Binding of AP-1 proteins to the DNA is determined not only by presence of the transcription factor but also by its redox state that is regulated by REF-1. In order to determine if the observed differences in AP-1 binding between monocytes and alveolar macrophages could be explained by differences in amounts of the protein REF-1, we obtained nuclear protein from both alveolar macrophages and monocytes from three different individuals. Western analysis for REF-1 was performed, and the results are shown in Fig. 5, A and B. In all three individuals there was significantly more REF-1 in the nuclei of monocytes than in the nuclei of alveolar macrophages. This experiment provided a possible explanation for the lack of AP-1 binding observed in PMA-treated alveolar macrophages. In order to make sure that treatment of the cells is not changing the amounts of REF-1, we evaluated REF-1 protein levels in both alveolar macrophages and monocytes that were treated with either LPS or PMA for 3 h. As shown in Fig. 6, neither LPS nor PMA altered the amounts of REF-1 in the cells.

Monocytes Have a Decreased Amount of REF-1 Following Differentiation into Macrophages—We next performed an experiment that links differentiation of monocytes to the defect in AP-1 DNA binding seen in alveolar macrophages. As an in vitro correlate of the in vivo differentiation of monocytes into alveolar macrophages, we evaluated the amount of REF-1 in the nucleus of differentiated monocytes and the ability of PMA to induce AP-1 binding in differentiated monocytes. In order to do this, we obtained blood monocytes and harvested nuclear protein from half the cells immediately after isolation. The remaining cells were put into culture with 10% human AB serum for 7 days. At the end of this time period, the cells morphologically resembled macrophages and nuclear protein was isolated. Fig. 7 shows that the process of differentiation into macrophage-like cells is associated with decreased amounts of REF-1. Fig. 8 is an AP-1 gel shift with PMA-treated nuclear proteins from monocytes compared with differentiated monocytes. The differentiated monocytes have lost their ability to increase AP-1 binding with PMA. There is more AP-1 binding in the control differentiated monocytes than in the control undifferentiated monocytes. This could just be a function of the difference between in vitro differentiation and in vivo differentiation. As a composite these two experiments show that with differentiation monocytes have reduced amounts of REF-1 and do not respond to PMA with increased AP-1 DNA binding.

REF-1 from Monocytes Can Increase AP-1 Binding in Nuclear Extracts from Alveolar Macrophages—The second experiment to link a defect in AP-1 binding to REF-1 was performed in alveolar macrophages. Both monocytes and alveolar macrophages were cultured with and without PMA, and nuclear protein was isolated. We next immunoprecipitated REF-1 from 20 μg of monocyte nuclear extract and added it to the macrophage nuclear proteins 15 min before addition of labeled probe and allowed to sit at room temperature. Fig. 9 shows that PMA-treated macrophages alone do not exhibit significant amounts of AP-1 DNA binding (2nd lane) and that the addition of monocyte REF-1 results in AP-1 binding (3rd lane). The amount of AP-1 binding in the control alveolar macrophage is a function of the variation found between individuals. Some people showed no AP-1 binding, and others showed a small amount of base-line binding. None of the individuals studied showed any increase in binding with the addition of PMA. We also performed a number of controls as follows: the 4th lane contains macrophage nuclear extract with monocyte protein precipitated with an isotype control antibody (rabbit IgG), the 5th lane contains macrophage nuclear protein with monocyte protein precipitated with an irrelevant antibody (α65, Santa Cruz Biotechnology), and the 6th lane contains immunoprecipitated monocyte REF-1 with no macrophage nuclear protein. Also shown on this blot is an AP-1 gel shift of the monocyte nuclear protein used for the immunoprecipitations. This figure shows that AP-1 proteins from PMA-treated alveolar macrophages can bind to DNA after monocyte REF-1 has been added.

DISCUSSION

This study shows that the defect in protein kinase C-induced AP-1 DNA binding activity seen in normal alveolar macro-
phages, compared with monocytes, is due to a relative lack of the redox protein, REF-1, and is not due to a lack of AP-1 proteins. We were able to reconstitute normal AP-1 DNA binding in alveolar macrophage nuclear extracts by adding REF-1 from monocytes. This was specific for AP-1, since the DNA binding activity of NF-κB was similar in alveolar macrophages and monocytes. This loss of REF-1 in alveolar macrophages may be due to the process of macrophage differentiation since monocytes lost REF-1 as they differentiated in vitro, into macrophage-like cells. To our knowledge, this study is the first description of normal cells with a defect in AP-1 binding caused by a lack of REF-1. AP-1 is an important regulator of expression of many genes. The relative lack of REF-1 and AP-1 DNA binding activity may explain, in part, the functional differences between alveolar macrophages and their precursors, monocytes.

REF-1 is a ubiquitous nuclear protein found in mammalian nuclear extracts. It was initially studied for its DNA repair function, which is found at the C-terminal portion of the molecule. The redox regulator, located close to the N terminus, is structurally and functionally distinct from the DNA portion of the molecule (35, 36). REF-1, itself, is controlled by redox modification of cysteines by the protein, thioredoxin (25).

REF-1 controls the DNA binding capacity of FOS and JUN proteins through conserved cysteine residues flanked by basic

**FIG. 4.** PMA induces increased c-FOS and c-JUN mRNA and protein in both monocytes and alveolar macrophages. Monocytes and alveolar macrophages were cultured with LPS (1 μg/ml) or PMA (100 ng/ml) for 3 h. A, whole cell RNA was obtained and run out on a 1.5% formaldehyde gel. A Northern blot was obtained and probed with 32P-labeled c-DNA, specific for c-FOS and c-JUN. This is an autoradiogram of the labeled blot. B, whole-cell protein from cells treated identically to the mRNA cells was isolated and run out on a 10% polyacrylamide-SDS gel. Western analysis was performed, and c-FOS and c-JUN were visualized using c-FOS- and c-JUN-specific antibodies and chemiluminescence. These are autoradiograms of the immunoactive bands.

**FIG. 5.** Monocytes contain increased amounts of the nuclear protein, REF-1, compared with alveolar macrophages. Nuclear protein was isolated from monocytes and alveolar macrophages immediately after isolation as described under “Materials and Methods.” Western analysis was performed on 20 μg/sample of nuclear protein from three individuals. REF-1 was visualized using an REF-1-specific antibody and chemiluminescence. A is an autoradiogram of the immunoactive bands. 1, 2, and 3 refer to the three individuals whose matching monocytes and alveolar macrophages were used for protein isolation. B shows a quantitation of the densitometry performed on the Western analysis, and statistical significance was evaluated using the mean gray level values and the Student’s t test. *p < 0.001 for monocytes compared with alveolar macrophages.

**FIG. 6.** LPS and PMA do not change the amounts of REF-1 in either alveolar macrophages or monocytes. Nuclear protein was isolated from monocytes and alveolar macrophages after 3 h of treatment with either LPS (1 μg/ml) or PMA (100 ng/ml). Western analysis was performed on 20 μg/sample of nuclear protein. REF-1 was visualized using an REF-1-specific antibody and chemiluminescence. This is an autoradiogram of the immunoactive bands.

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amino acids found in the DNA binding domain of all FOS- and JUN-related proteins (37, 38).

There are only a few previously described instances of modulation of the expression of REF-1. Fung et al. (26) showed that asbestos caused an increase in REF-1 gene expression in mesothelial cells. Asai et al. (27) showed that thyrotropin increased REF-1 mRNA and protein in rat thyroid FRTL-5 cells, and Suzuki et al. (28) showed that human chorionic gonadotropin increased REF-1 in murine Leydig cells. In an animal model, Gillardon et al. (29) showed that global ischemia induced by cardiac arrest increased expression of REF-1 in the rat hippocampus. All of these studies are cases in which an intervention results in an increase in REF-1 expression.

In contrast to these studies, Robertson et al. (30) showed that induction of apoptosis in HL-60 cells results in down-regulation of REF-1. Walton et al. (31) evaluated rat brains following ischemia and found that apoptotic cells had a significant decrease in amounts of REF-1. One possible explanation for our data is that the alveolar macrophages are undergoing apoptosis. This is not likely since all studies of normal human alveolar macrophages reported to date showed that the cells must be exposed to a toxic stimulus to trigger apoptosis. We showed in a number of studies that alveolar macrophages are not activated, unless they are exposed to endotoxin or another stimulus in vitro (5, 9–11, 32, 33). Our studies evaluated REF-1 immediately after the cells were obtained from normal volunteers. These observations are supported by observations of Bingisser et al. (34) who showed that unstimulated normal human alveolar macrophages do not undergo apoptosis but that apoptosis could be induced in human alveolar macrophages by high levels of endotoxin.

These studies are the first to show that a normal cell can be deficient in REF-1 and that this can result in a defect in AP-1 expression. These studies also suggest that it is the process of differentiation that results in the decreased levels of REF-1 in alveolar macrophages. The findings that we describe in this study may explain some of the functional differences between alveolar macrophages and their precursors blood monocytes.

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