Biochemical and Biophysical Research Communications **269,** 284–289 (2000)
doi:10.1006/bbrc.2000.2282, available online at http://www.idealibrary.com on IDEAL®

Induction of Leukemia Cell Differentiation and Apoptosis by Recombinant P48, a Modulin Derived from *Mycoplasma fermentans*[1]

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Received February 3, 2000

P48 is a 48-kDa monocytic differentiation/activation factor which was originally identified in the conditioned medium of the Reh and other leukemia cell lines and has recently been shown to be a *Mycoplasma fermentans* gene product. Previously, conditioned medium P48 has been shown to induce differentiation of HL-60 (human promyelocytic leukemia) cells. Recently our laboratory isolated cDNA clones for P48 from Reh cells and genomic clones from *Mycoplasma fermentans* and expressed the recombinant protein as a maltose binding protein (MBP) fusion protein in *E. coli*. In this report we present the initial characterization of this recombinant P48 fusion protein (rP48-MBP). We show that rP48-MBP induces differentiation of HL-60, U937 (human histiocytic lymphoma), and M1 (mouse myeloid leukemia) cell lines. Interestingly, rP48-MBP also induces apoptosis of U937 and HL-60 cells as assessed by terminal transferase (TUNEL) assays. This is the first report of induction of apoptosis by a *Mycoplasma* gene product. P48 is a *Mycoplasma*-derived immunomodulatory molecule which has differentiation and apoptosis-inducing activities and may be important in the pathophysiology of *Mycoplasma* infections. The recombinant protein may be useful in studying the mechanisms of differentiation, cytokine production, and apoptosis in malignant and nonmalignant hematopoietic cells.

A number of cytokines have now been identified which modulate growth, differentiation, and functional activity of hematopoietic and lymphoid cells (1–4). In addition, other factors such as vitamins (5, 6) and a number of microbial products collectively known as modulins (reviewed in reference 7) are also known to modulate the functions of these cells. Modulins which have been shown to activate leukocytes include LPS (8), TSST-1 (9), erythrogenic toxins A and C (10), and several *Mycoplasma* products (11–15).

Our laboratory has previously identified, purified, and extensively characterized a 48-kDa protein (termed P48) from human leukemic cell line conditioned media which induced hematopoietic differentiation and activation of mononuclear phagocytes (16–20). Recently, we cloned the gene encoding for this factor and demonstrated that it is a *Mycoplasma fermentans* gene product which is likely to be a lipoprotein (21). We have also reported that the P48 gene may be specific for *M. fermentans* since it was not detected in a panel of other *Mycoplasma* species tested (22). Interestingly, antigenic cross-reacting protein was detected in some, but not all *Mycoplasma* species (22).

Using the cloned cDNA for P48 we have expressed and purified the recombinant P48 protein as a maltose binding protein (MBP) fusion protein. In this report, we present initial *in vitro* studies examining growth, differentiation, and apoptosis activities of recombinant P48 on human and mouse myeloid cell lines.

MATERIALS AND METHODS

Reagents. Tissue culture media, antibiotics, and Trizol RNA extraction reagents were obtained from GIBCO/BRL (Grand Island, NY). Reagents for cDNA synthesis were purchased from Promega (Madison, WI).

Cells and cell lines. The Reh (human pre-B leukemia), HL-60 (human promyelocytic leukemia), U937 (human histiocytic lymphoma), and M1 (mouse myeloid leukemia) cell lines were maintained in RPMI-1640 medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (F10 medium). All cultures were maintained at 37°C in an atmosphere of 5% CO_2/95% air. The Reh cell line has been maintained in our lab for

Abbreviations used: H7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; MBP, maltose-binding protein; rP48, recombinant P48; RT-PCR, reverse transcriptase-polymerase chain reaction; TUNEL, TdT-mediated dUTP Nick-End Labeling.

[1] Supported by NIH Grants CA-58205 and CA-72591.

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0006-291X/00 $35.00

several years and was recently shown to be contaminated with *Mycoplasma fermentans* or a closely related strain (21, 22).

Preparation of recombinant rP48-MBP. The P48 cDNA clone 22 (21) was ligated into the pMAL-C2 expression vector (New England Biolabs, Beverly, MA), transformed into *E. coli*, grown to exponential phase and induced with 1 mM IPTG. Bacterial lysates were then prepared and submitted to amylose affinity chromatography as described by Maina *et al.* (23). Purified proteins were analyzed by SDS-PAGE and Western blot analysis as described (21), probing with 1:200 rabbit anti-P48 antiserum or normal rabbit serum (control) followed by 1:5000 alkaline phosphatase-conjugated goat anti-rabbit Fc-Ig (Promega).

Cell culture with recombinant P48-maltose binding protein (rP48-MBP). Cell line cells were washed in F10 medium, resuspended at 10^6 cells/ml, and cultured 3 days with various concentrations of rP48-MBP or control MBP in 24-well (16-mm) tissue culture plates (Corning, Corning, NY). Cells were then removed by vigorous pipetting, washed, and further analyzed as described below and in figure legends.

Proliferation assays (^{3}H-thymidine incorporation). This assay has been described previously (19, 20). In brief, 100 μl of cells (1×10^5 cells/ml) were placed in triplicate flat bottom microtiter wells (Corning, Corning, NY) and cultured with 100 μl of various dilutions of rP48-MBP or MBP control for three days, after which 0.5 μCi/well [^{3}H]thymidine (78 Ci/mmol; ICN, Irvine, CA) was added. After an additional 6 hours of culture, incorporated radioactivity was harvested using a Dynatech Minimash 2000 cell harvester (Dynatech, Alexandria, VA), and filters were placed in scintillation vials and counted with an aqueous-based scintillation fluid (Ecolume, ICN).

Indirect immunofluorescence and flow cytometry. This assay has been previously described (19, 20). In brief, 10^6 cultured cells were washed, incubated with CD11b monoclonal antibody (Mo1, Coulter, Hialeah, FL) or control normal mouse IgM kappa and washed further. After a second incubation with fluorescein-labeled goat anti-mouse immunoglobulin (Biosource International, Camarillo, CA) and further washing, cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Northern analysis. Procedures were performed as previously described (21). RNA was extracted from cultured cells using Trizol reagent and then electrophoresed on 1% agarose gels in 20 mM MOPS (pH 8.0), 1.1% formaldehyde, 1 mM EDTA. Hybridization was carried out at 41°C for 16 hours with ^{32}P-labeled heat-denatured TNF-α cDNA probe (17, 24) (10^6 cpm/ml, specific activity $>10^8$ cpm/μg DNA) in prehybridization buffer consisting of 50% formamide in 2× Denhardt's solution (0.04% w/v Ficoll 400, 0.04% polyvinylpyrrolidone, 0.04% bovine serum albumin), followed by washing once (15 min, 41°) in 3× SSC and then further washing up to high stringency (0.1× SSC, 41°). The blots were then exposed to Biomax MS film (Eastman Kodak, Rochester, NY) and developed.

TdT-mediated dUTP nick-end labelling (TUNEL) assay of apoptosis. The TUNEL Apoptosis Detection System (Promega, Madison, WI) was used. Cells (2×10^6/2 ml/well) were placed in 24-well (16-mm-diameter) tissue culture plates and cultured in the presence of various agents or control F10 medium alone for three days. Cells were then collected, washed with PBS, fixed (20 min, 4°C) with 1% methanol, and permeabilized (5 min, 4°C) with 0.2% Triton X-100 in PBS. Intracellular DNA fragments were then labeled by exposing cells (37°C, 1 hour) to fluorescein-12-dUTP and terminal deoxynucleotidyltransferase. The reaction was stopped by adding 20 mM EDTA, followed by washing the cells and staining with 5 μg/ml propidium iodide containing 250 μg/ml DNase-free RNAse. Cells were then analyzed for both green and red fluorescence on a FACScan flow cytometer (Becton-Dickinson).

RESULTS

Growth inhibition of HL-60 human promyelocytic leukemia, U937 human histiocytic lymphoma, and M1 mouse myeloid leukemia cell lines by recombinant P48. As hematopoietic cells differentiate they typically undergo slowing of growth and then growth arrest (25, 26). We have previously reported that P48 purified from Reh cell line conditioned medium markedly inhibits the growth of a panel of human and mouse malignant cell lines and induces hematopoietic differentiation (19, 20). Recently we cloned and expressed the gene for P48, and as a prelude to examining *in vivo* effects including potential differentiation therapy of leukemia, we wished to examine the growth, differentiation, and apoptosis properties of the recombinant molecule *in vitro.* We have examined several recombinant protein expression systems, and to date we have found that expressing rP48 as a maltose-binding protein (MBP) fusion protein in the pMAL system leads to adequate production of biologically active material which can be purified by amylose affinity chromatography. First, we examined the effect of rP48-MBP fusion protein on the growth of human and mouse myeloid cell lines using a standard [^{3}H]thymidine incorporation assay (Fig. 1). We found that rP48-MBP inhibited proliferation >50% when cultured with cells for three days at 1–2 μg/ml, in contrast to control MBP which had no appreciable effect on proliferation at these concentrations. Time course and dose–response studies (not shown) revealed a dose-dependent inhibition of proliferation detectable after as little as one day of culture in the presence of rP48-MBP. The order of sensitivity of these cell lines to rP48-MBP was: M1 > U937 ~ HL-60. Since rP48-MBP fusion protein is composed of 30% rP48 and 70% MBP by weight (polypeptide molecular weights 18 kDa and 42 kDa, respectively) (21), rP48 has approximately the same biologic specific activity as P48 isolated from Reh cell line conditioned medium. Since [^{3}H]thymidine incorporation is a measure of DNA synthesis, in order to further analyze cell growth we also counted cells after three days of exposure to rP48-MBP and found that cell numbers paralleled [^{3}H]thymidine incorporation data (not shown).

Stimulation of TNF-α mRNA. As immature hematopoietic cells differentiate to mature monocytes or polymorphonuclear leukocytes, they demonstrate morphologic changes, develop functional activities, and undergo cell surface changes including modulation of surface antigens (27, 28). One functional change that occurs is that mature monocytes accumulate mRNAs for a number of inflammatory cytokines, including TNF-α and IL1 (29, 30). In order to examine the differentiation activity of rP48-MBP we cultured HL-60 cells for three days in the presence and absence of rP48-MBP and control MBP, followed by extraction of

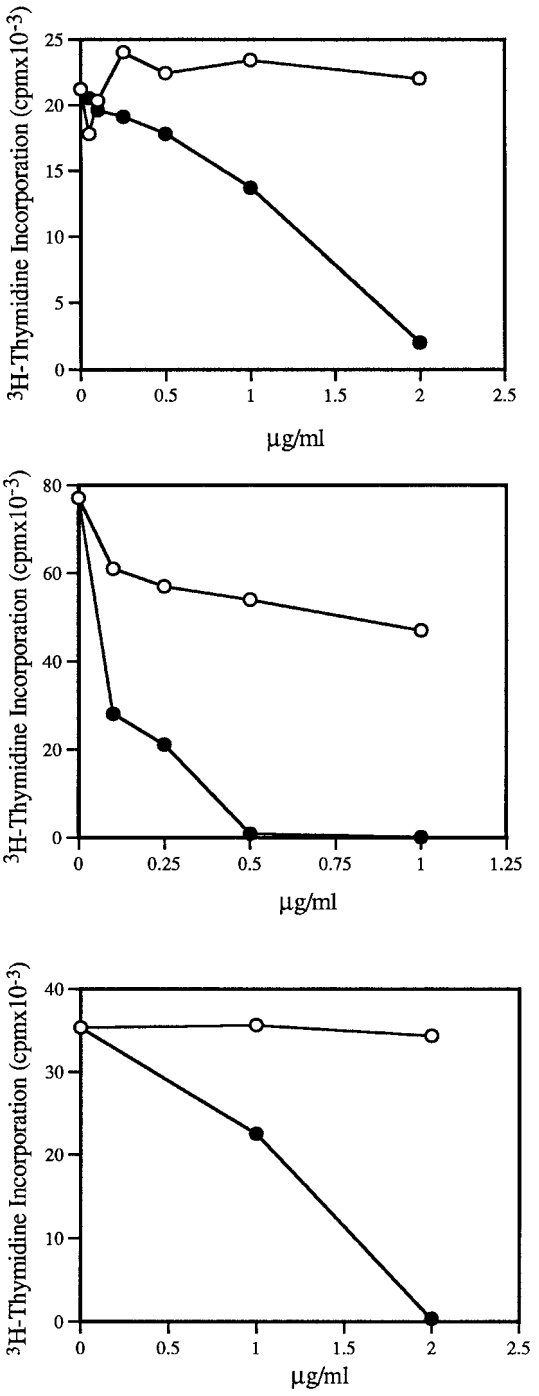

FIG. 1. Effect of rP48-MBP on proliferation of human and murine hematopoietic cell lines. Cells (10^4/well) were cultured in 96-well flat-bottom microtiter plates for 72 hours in the presence of various concentrations of purified rP48-MBP (closed circles) or MBP (open circles) followed by 6 hour culture with [^{3}H]thymidine and assay of incorporation into DNA. Upper panel, HL-60 cells; middle panel, M1 cells; lower panel, U937 cells.

cellular RNA and assay of TNF-α mRNA by Northern blot analysis. Figure 2 shows that HL-60 cells cultured in medium alone or control MBP protein had a low, nearly undetectable level of TNF-α mRNA whereas cells cultured in the presence of rP48-MBP or the strong differentiation agent PMA had markedly increased levels of this transcript. In data not shown, we found similar results when TNF-α mRNA levels were assayed by reverse transcriptase-polymerase chain reaction (RT-PCR) using TNF-α specific primers (17). We also examined Giemsa-stained cytocentrifuge preparations of HL-60 cells cultured in the presence of rP48-MBP and controls and found that cells exhibited morphologic changes (lobulation of nucleii and clumping of chromatin, increased size, vacuolation of cytoplasm) suggestive of differentiation along the monocyte–macrophage pathway (not shown).

Induction of expression of CD11b (Mac-1/Mo1) surface antigen on HL-60 cells. The expression of CD11b, a receptor for the third component of complement (complement receptor type three or CR3) (31), is known to increase as cells differentiate along the hematopoietic pathways toward monocytes or granulocytes (32). As a further analysis of the differentiation activity of rP48-MBP, we measured expression of the surface antigen CD11b after exposure of HL-60 cells to recombinant proteins for 3 days. When assayed by indirect immunofluorescence/flow cytometry, we found that CD11b expression increased substantially when HL-60 promyelocytes were exposed to rP48-MBP, in contrast to control MBP which had little effect (Table 1). Both the number of cells expressing CD11b and the number of CD11b antigenic sites per cell (mean fluorescence intensity) increased after exposure to rP48-MBP.

Induction of apoptosis. Programmed cell death or apoptosis is a process in which environmental stimuli activate a series of events leading to fragmentation of DNA and death of the cell (reviewed in reference 33). Studies in transgenic mice and other systems have

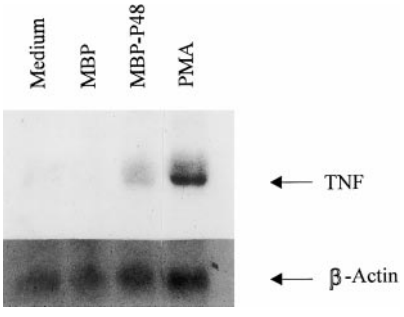

FIG. 2. Northern analysis of TNF-α mRNA expression in HL-60 cells cultured with rP48-MBP. In the top panel, the blot was probed with ^{32}P-labeled TNF-α cDNA probe, and in the bottom panel the same blot was probed with ^{32}P-labeled β-actin cDNA probe. Each lane was loaded with 10 μg total cellular RNA. Arrows along the right margin indicate the ~18S TNF-α and β-actin mRNA hybridization bands. Lane 5, which contained RNA from PMA-stimulated cells, contained more RNA than the other lanes, as indicated by the greater β-actin hybridization signal.

TABLE 1

Expression of CD11b Surface Antigen on Cultured HL-60 Cells[a]

Addition	% Fluorescence			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Medium	3	2	2	3 (54)
MBP (1 μg/ml)	12	17	21	18 (112)
rP48-MBP (1 μg/ml)	41	40	48	44 (179)
PMA (20 ng/ml)	—	16	2	33 (288)
P19 (5 μg/ml)	—	12	11	11 (104)

[a] HL-60 cells were cultured for 3 days in the presence of medium alone, MBP, rP48-MBP, PMA, or P19 (a 19-kDa control protein purified from bacterial lysates). Cells were then washed and analyzed for CD11b surface antigen expression by indirect immunofluorescence/flow cytometry as described under Materials and Methods. Data from four separate experiments are presented. In Experiment 4, the numbers in parentheses represent mean log fluorescence.

suggested that regulation of apoptosis is an important part of normal growth and development (34, 35). We therefore examined the effect of rP48-MBP on apoptosis of HL-60 and U937 cells (Fig. 3). In order to assay apoptosis, we used the TdT-mediated dUTP Nick-End Labeling (TUNEL) assay, in which TdT is used to add fluorescein-12-dUTP to 3′ ends of fragmented DNA, followed by flow cytometry to quantitate numbers of apoptotic cells (36). We found minimal apoptosis of HL-60 cells following 3-day exposure to 1 μg/ml of rP48-MBP whereas appreciable numbers (~40%) of U937 cells were apoptotic after similar exposure. At 2 μg/ml rP48-MBP, we observed >80% apoptosis of U937 and a small amount (about 15% compared to controls) of apoptosis of HL-60. In data not shown, we have confirmed these apoptosis studies by staining cells with Hoechst Dye 33258, which revealed fragmented condensed chromatin, a morphological feature of apoptotic cells (37).

DISCUSSION

Our laboratory has previously identified, purified, and extensively characterized a 48-kDa protein with monocytic differentiation and activation properties termed P48 (16–20). P48 was initially identified in the conditioned media of the Reh human leukemic cell line, and later shown to be present associated with cell membranes (18). The biologic activities of this factor include induction of monocytic differentiation and cytolytic activity in the HL-60 (human promyelocytic leukemia) cell line, growth inhibition towards a large panel of other human and mouse tumor cell lines, and induction of IL-1 and TNF-α mRNA and protein by human peripheral blood monocytes. Recently we cloned the gene encoding for this factor and demonstrated that it is a *Mycoplasma fermentans* gene product which is likely to be a lipoprotein (21).

We have expressed the P48 gene as a maltose binding protein (MBP) fusion protein and have purified the recombinant protein. In the current study we now report the initial characterization of the growth and differentiation activities of this recombinant fusion protein. We show that rP48-MBP fusion protein has growth and differentiation properties including inhibition of proliferation of human (U937, HL-60) and mouse (M1) hematopoietic cell lines, stimulation of expression of CD11b surface antigen, and induction of TNF-α mRNA.

Recombinant P48 was produced in *E. coli*, and such prokaryotic expression systems are known to express recombinant proteins with little if any post-

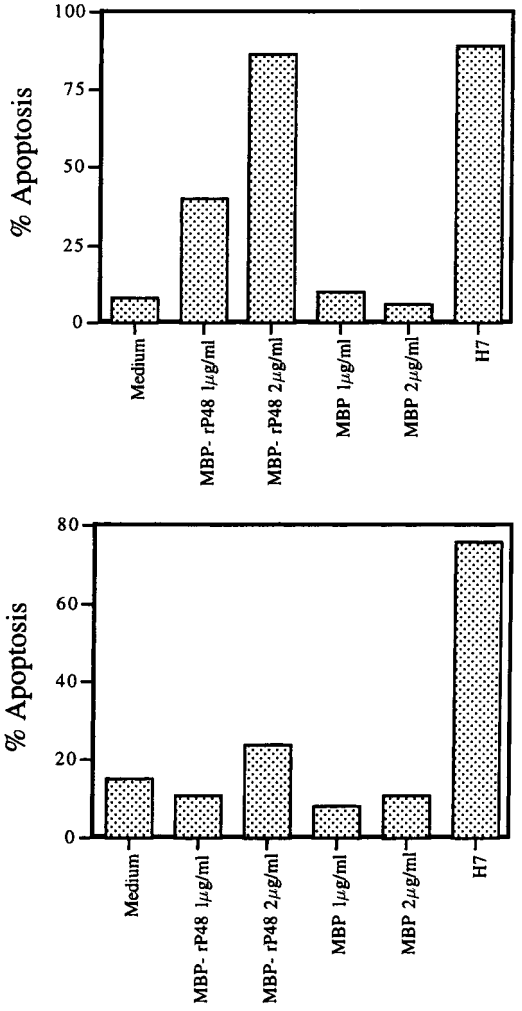

FIG. 3. Apoptosis of HL-60 and U937 cells. HL-60 (lower panel) or U937 (upper panel) cells were cultured 3 days in the presence of 1 or 2 μg/ml rP48-MBP, 1 or 2 μg/ml MBP, or 50 μM H7, a strong inducer of apoptosis (positive control). Cells were then washed, fixed, submitted to TUNEL assay as described under Materials and Methods, and analyzed on a FACScan flow cytometer. This was a representative experiment.

translational modification (e.g., glycosylation, lipidation). This along with the molecular weight of rP48 (18 kd) suggest that rP48 lacks most or all of the lipid and other post-translational modifications found in the native 48 kd molecule. The data reported here suggest that at least some of the growth and differentiation activities of P48 are due to the polypeptide portion of the molecule, but do not rule out the possibility that some of the biologic activities may be associated with lipid and/or carbohydrate components of the native molecule.

In addition to examining cell growth (as assessed by [^{3}H]thymidine incorporation into DNA and cell number) and differentiation, we also examined the effect of rP48-MBP fusion protein on apoptosis. We found that there was a modest increase in apoptosis of HL-60 cells up to 2 μg/ml rP48-MBP, and a striking increase in apoptosis of U937 cells after 3 day exposure to the recombinant fusion protein. These studies suggest that rP48-MBP is capable of inducing terminal differentiation of leukemia and lymphoma cells. The reason(s) for the differences observed in apoptosis of HL-60 and U937 cells are not clear, but are currently under investigation.

A large number of cytokines and other factors have now been identified which modulate growth, differentiation, and functional activity of hematopoietic cells. A number of cytokines (1–4) and vitamins (5, 6) are known to induce differentiation and activate leukocytes. Several microbial products collectively known as modulins (7) including LPS (8), TSST-1 (9), erythrogenic toxins A and C (10), and a variety of *Mycoplasma* products (11–15) have been shown to activate leukocytes *in vitro* but have not been shown to induce differentiation or apoptosis. We believe that P48 is the first *Mycoplasma*-derived factor which has been shown to induce differentiation and apoptosis of mammalian cells. These studies suggest that *Mycoplasma*-derived P48 may have important effects on apoptotic pathways which are known to be important in both T cell and B cell mediated immune responses (33). Thus, immunomodulatory effects of P48 may potentially include cytokine induction, activation of mononuclear phagocytes, induction of hematopoietic differentiation, and modulation of hematopoietic/lymphoid cell apoptosis.

P48 is an interesting molecule with immunomodulatory and hematopoietic differentiation activities and may be important in the pathophysiology of *Mycoplasma* infections. The studies reported here with recombinant P48 were done as part of an initial characterization of *in vitro* activities prior to examining *in vivo* effects in mice, including potential differentiation therapy of leukemia. Recombinant rP48-MBP fusion protein should be a useful agent to examine the mechanisms of hematopoietic differentiation, cytokine production, and apoptosis, and may be a potentially useful new agent to treat leukemia or as an immunomodulatory agent in cancer and other diseases.

ACKNOWLEDGMENTS

We thank Dr. Joseph Fuhr and Mr. Richard Andrews for performing the flow cytometry analyses.

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