Anti-Cancer Drugs, 1, pp. 83–87

Short communication

Partial biochemical characterization of spiroplasma membrane component inducing tumor necrosis factor$_\alpha$

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We have recently found that membranes of *Spiroplasma* spp. strain MQ-1 (hereafter referred to as MQ-1) induce both tumor necrosis factor$_\alpha$ (TNFα) secretion by bone marrow macrophages and blast transformation of lymphocytes via a mechanism different from that operated by bacterial lipopolysaccharide (LPS). This report presents evidence indicating that the MQ-1-derived membrane component(s) which activates bone marrow macrophages to secrete TNFα is, at least in part, protein. This conclusion is supported by our findings that TNFα secretion was reduced following exposure of MQ-1 membranes to elevated temperatures, extreme acidic pH treatment and incubation with protease K or pronase. Furthermore, following lipid extraction of MQ-1 membranes, most of both induction of TNFα secretion and blast transformation activities appeared in the 'protein' fraction. When membranes were chromatographed on a phenyl–Sepharose column, two major peaks were obtained, one containing most of the TNFα induction activity and the other the mitogenic activity. Neither peak coeluted with the peak of bulk membrane lipids. The possibility that the spiroplasma membrane component inducing TNFα secretion is acylated protein is discussed.

Key words: Tumor necrosis factor, spiroplasma, membrane components.

Introduction

Spiroplasmas (class *Mollicutes*, family *Spiroplasmataceae*) are motile wall-less microorganisms characterized by a helical morphology. These organisms are widely distributed in arthropods and plants.[1] The cell membrane of spiroplasmas, like that of mycoplasmas, is built of protein and lipid.[2] Limited information has been published regarding the mitogenic activity of spiroplasmas,[3] and nothing actually about their ability to activate macrophages.

We have recently shown[4] that membranes of MQ-1, a spiroplasma isolated from hemolymph of a vespid wasp,[5] are very potent inducers of tumor necrosis factor$_\alpha$ (TNFα) secretion and blast transformation. Our studies indicated that MQ-1 membranes induced TNFα secretion and tumor cytolysis by bone marrow macrophages via a mechanism different from that operated by bacterial lipopolysaccharide (LPS). These membranes also induce TNFα secretion by human monocytes.[4]

Preliminary *in vivo* studies carried out in our laboratory indicated that MQ-1 membranes were less toxic in rodents than LPS and showed some promising antitumor activity (unpublished data). Therefore, we have started purification and characterization of the MQ-1 membrane components inducing TNFα secretion and/or blast transformation for evaluating their therapeutic potential in treatment of malignant diseases. Results

This study was supported by the Ernst David Bergmann Fund of the Hebrew University, Jerusalem, and by a grant from the United States–Israel Binational Agriculture Research Development Foundation.

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of such preliminary biochemical characterization presented herein indicate that the MQ-1 membrane component(s) inducing TNFα secretion is, at least in part, protein.

Materials and methods

Mice

Six to eight week old, inbred C57BL/6 pathogen-free mice were obtained from Jackson Laboratory Animal Resources, Bar-Harbor, ME.

Bone marrow macrophage cultures

C57BL/6 bone marrow-derived macrophages were cultivated for 9–16 days before use as previously described.[6,7]

Target cell cultures

The TNFα sensitive cell line, BALB/c CL.7, a continuous fibroblast cell line, was cultivated as described.[8] All cultures, including murine-derived bone marrow macrophages, were shown to be mycoplasma-free, as assayed by cultivation on Hayflick medium.[9]

Cultivation of MQ-1 cells and isolation of membranes

Spiroplasma spp. strain MQ-1 (hereafter referred to as MQ-1) was kindly provided by Dr RF Whitcomb, Agricultural Research Center, US Department of Agriculture, Beltsville, MD. The organisms were grown at 32°C for 48 h in a modified medium of Saglio *et al.*,[10] supplemented with 10% (v/v) horse serum (Biolab, Israel). The cells were harvested, membranes were isolated by osmotic lysis,[11] washed and resuspended in 0.25 M NaCl in 0.05 M Tris–HCl buffer pH 7.5 and kept at −70°C until use. Membrane protein, being easy to determine, was chosen as a parameter of reference and was determined according to Lowry *et al.*[12] using bovine serum albumin as a standard.

Lipid extraction from MQ-1 membranes

Lipid extraction from [^{3}H]palmitate-labeled (5 μCi/100 ml medium, palmitic acid [9,10-^{3}H], specific activity 60 Ci/mM, American Radiolabeled Chemicals, Inc., St Louis, MO) MQ-1 membranes were carried out by slight modification of the chloroform:methanol method.[13] In short, 1 volume of MQ-1 membrane preparation (2 mg protein/ml) was mixed with 3.5 volumes of methanol and subsequently 3.5 volumes of chloroform were added. The mixture was incubated for 30 min at room temperature with occasional mixing. The reaction tubes were then centrifuged in an Eppendorf centrifugation for 3 min and the protein pellet was separated from the supernatant (referred to as 'protein' fraction). The chloroform:methanol mixture was evaporated to dryness under nitrogen (referred to as 'lipid' fraction) and both the 'protein' and the 'lipid' fractions were then solubilized in Chaps (2 mg/ml) and sonicated for 2 h in a sonication bath.

Phenyl–Sepharose column

[^{3}H]palmitate labeled MQ-1 membranes were dissolved in 7 M guanidine–HCl (Sigma, USA), then diluted 1:1 with 0.05 M Tris–HCl buffer pH 8.0 (referred to as 'buffer'), and loaded on a phenyl–Sepharose column (20 mm × 5 mm, Pharmacia), equilibrated with 1 M NaCl in buffer. The column was eluted with 3 ml of 1 M NaCl in the buffer followed by buffer alone, 20% ethylene glycol, 40% ethylene glycol (Eastman Organic Chemicals, NY), 20% acetonitrile (Sigma), all of them in the buffer solution. Then, 40, 50, 60, 70 and 90% acetonitrile in distilled water containing 0.05% trifluoroacetic acid (Sigma). Samples diluted 1:100 were assayed for induction of TNFα secretion and blast transformation.

Determination of TNFα activity

Twenty-four hour supernatants of activated bone marrow macrophages were harvested and assayed for TNFα activity in a biological assay as previously described,[14] except that actinomycin-D treated BALB/c CL.7 cells were substituted for L929 cells as targets. The units given for the TNFα titers, S_{50}, are the reciprocal values of the supernatant dilution that would cause lysis of 50% of the CL.7 cell monolayer. A logit transformation computer program was used for the calculations.

Table 1. Effect of elevated temperatures and extreme pH treatment on MQ-1 membrane activities*

Experiment A: Temperature treatment		Experiment B: pH treatment	
Temperature	TNFα titer (S_{50})	pH	TNFα titer (S_{50})
Control	44 920	Control	22 380
60°C	34 840	1.5	7 030
80°C	1 580	9.0	15 540
100°C	440	12.0	39 270

* Macrophages in each experiment were activated with MQ-1 membranes (5 μg protein/ml). Membranes were exposed to elevated temperatures for 15 min. Membranes were exposed to extreme pH treatment for 30 min, followed by neutralization.

Evaluation of the mitogenic response

The method is based upon that previously described.[15] Splenic cells alone, or with activating agents, were incubated for 72 h. The cells were then pulsed for an additional 5 h with 0.5 μCi [^{3}H]thymidine per well (S.A. 5 Ci/mmol; New England Nuclear Corp., Boston, MA). The cells were harvested and the [^{3}H]thymidine uptake was determined. Results were the means of triplicate and quadruplicate assays ± standard deviation (SD).

Results

Effects of elevated temperatures, extreme pH values and proteases on MQ-1 membrane activities

Membranes of MQ-1 (1 mg/ml) were exposed to 60, 80 and 100°C for 15 min and subsequently diluted and assayed for their ability to activate bone marrow macrophages to secrete TNFα and induce blast transformation of splenic lymphocytes. Table 1 shows that TNFα titers were markedly reduced with elevated temperatures. Thus, a reduction of 99% in TNFα titers was observed following activation by MQ-1 membranes previously subjected to 100°C. The mitogenic activity of MQ-1 membranes, however, was more stable at elevated temperatures, with about 30% of this activity still retained after heating the membrane preparation to 100°C (data not shown).

Thirty minute exposure of MQ-1 membranes (1 mg/ml) to extreme pH values (1.5 and 12), followed by neutralization, resulted in a pronounced decrease (70%) in TNFα titers at the extreme acidic pH, while enhanced TNFα titers were induced by membranes subjected to pH 12 treatment (Table 1). The mitogenic activity was only slightly reduced so that 73% of activity was still retained even after exposure to pH 1.5 (data not shown).

Incubation of MQ-1 membranes (1 mg/ml) with protease K (50 μg/ml) and pronase (50 μg/ml) for 2 h at 37°C, prior to bone-marrow macrophage activation, resulted in 80 and 60% reduction in the secreted TNFα titers, respectively (data not shown).

TNFα induction and mitogenic activity of membrane-derived lipid and protein fractions

Both induction of TNFα secretion and blast transformation (Table 2) were exclusively exhibited by the protein enriched fraction, containing 75% membrane protein compared to intact membrane, and only traces (5.9%) of membrane lipids. Chaps,

Table 2. TNFα induction and mitogenic activity of lipid- and protein-enriched fractions derived from MQ-1 membranes

Activating agent	Chemical composition		TNFα titer (S_{50}) (sample dilution)		Mitogenic effect (cpm ± SD) (sample dilution)
	Lipid (%)	Protein (%)	(1:1 600)	(1:16 000)	(1:1 600)
Intact membranes	100.0	100.0	>118 100*	12 400	35 261 ± 3 443
Protein fraction	5.9	75	>118 100*	7 320	36 674 ± 3 773
Lipid fraction	81.0	21	5	0	5 941 ± 382
Chaps (2 mg/ml)	—	—	0		3 008 ± 390
Medium (control)	—	—	0		2 786 ± 362

* TNFα titer was too high to calculate since it did not reach S_{50}.
% lipids = (cpm of [^{3}H] palmitate labeled tested sample)/(cpm of [^{3}H] palmitate labeled intact membranes) × 100.
% protein = (protein of tested sample)/(protein of intact membranes) × 100.

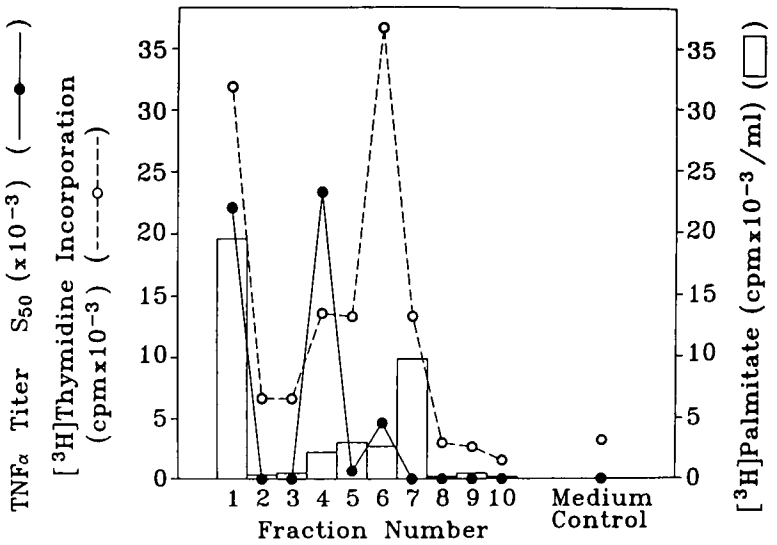

Figure 1. Elution of MQ-1 membrane activities from a phenyl–Sepharose column. [^{3}H]palmitate labeled MQ-1 membranes solubilized by guanidine–HCl in 0.05 Tris–HCl buffer pH 8.0 were loaded on a phenyl–Sepharose column equilibrated with 1 M NaCl in buffer. The column was eluted by 3 ml of 1 M NaCl in buffer, buffer alone, 20% and subsequently 40% ethylene glycol, 20% acetonitrile (all in the buffer solution) and then 40, 50, 60, 70 and 90% acetonitrile in distilled water containing 0.05% trifluoroacetic acid. The elution pattern of lipid was followed by determining the radioactivity in the fractions (cpm/ml of [^{3}H]palmitate) (□). TNFα titers, S_{50}, (–●–) and mitogenic activity (cpm of [^{3}H]thymidine incorporated into splenic lymphocytes) (–○–) induced by each sample (diluted 100-fold) were evaluated.

the solubilizing agent, affected neither TNFα induction nor blast transformation.

Partial purification of MQ-1 membrane activities by column chromatography

Considering the hydrophobic nature of spiroplasma membrane proteins[16,17] a phenyl–Sepharose column was employed for further purification. MQ-1 membrane, solubilized in guanidine–HCl were loaded on a phenyl–Sepharose column, and the elution was carried out as described in Materials and methods. Figure 1 shows that a major peak of TNFα induction activity and a minor peak of mitogenic activity (fraction 4) were coeluted from the column by 40% ethylene glycol (pH 8), whereas a minor peak of TNFα induction activity and a major peak of mitogenic activity (fraction 6) were coeluted by 40% aceonitrile (pH 4). Most of the membrane lipids were eluted by 50% acetonitrile, low pH (fraction 7).

Preliminary analytical chromatography on Superose 12, a gel filtration column (Pharmacia, bed size 10 × 300 mm), was carried out with MQ-1 membranes solubilized by Chaps (1.5 mg/ml). Elution with 0.05 M NaCl in Tris–HCl buffer pH 7.5, containing 0.05% Chaps, revealed two peaks of TNFα induction activity. One peak of high molecular weight (>68,000 daltons) and a low molecular weight peak (~15,000 daltons) (data not shown).

Discussion

Our studies present evidence, showing for the first time, that a spiroplasma membrane-derived component(s) which activated bone marrow macrophages to secrete TNFα is, at least in part, protein. This conclusion is supported by our observation that the TNFα induction activity was markedly reduced when exposed to elevated temperatures, extreme acidic pH and proteases prior to activation of bone marrow macrophages. The increase in TNFα induction activity at pH 12 may be due to exposure of the active membrane components upon saponifi-

cation of the membranes at the alkaline pH range.

Moreover, following lipid extraction of MQ-1 membranes, both TNFα induction and mitogenic activities resided exclusively in the 'protein' fraction. These results are compatible with the elution pattern obtained following further purification of solubilized MQ-1 membranes, on a phenyl–Sepharose column. Thus, none of the major peaks representing either the TNFα induction activity or the mitogenic activity coeluted with the peak of membrane bulk lipids.

The hydrophobicity of the eluted proteins in our study may be due either to high levels of hydrophobic amino acids or to the presence of covalently bound fatty acids. Indeed, it has been reported that sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *Spiroplasma melliferum* revealed 51 polypeptides by Coomassie blue staining of which 21 were acylated predominantly with myristic and palmitic chains. Spiralin, the major membrane protein of this bacterium, was shown to be acylated.[16,17] These data, as well as ours, raise the possibility that acylated polypeptides may be the active agents inducing TNFα secretion and blast transformation.

The elution pattern from the phenyl-Sepharose column suggests that different molecules are responsible for each of the spiroplasma membrane activities: induction of TNFα secretion and blast transformation. Our current studies are directed to further purify and identify the active components in order to evaluate their therapeutic potential in treatment of malignant diseases.

Conclusions

We have showed that spiroplasma membrane-derived component(s) which activates bone marrow macrophages to secrete TNFα is, at least in part, protein.

It seems that different components are responsible for each of the MQ-1 membrane activities: induction of TNFα secretion and blast transformation. Nevertheless, the existence of a membrane component capable of inducing both activities cannot be excluded at this stage of research.

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(Received 20 August 1990; accepted 30 August 1990)