Thymomodulin increases release of granulocyte-macrophage colony stimulating factor and of tumour necrosis factor in vitro

B. Balbi*, M.T. Valle**, S. Oddera†, E. Balleari**, F. Manca**, G.A. Rossi†, L. Allegra*


ABSTRACT: To evaluate the effects of thymomodulin (TMD), a thymic biological response modifier derived from calf thymus, on the release of various cytokines involved in the lung immune reactions, human alveolar macrophages (AM) and peripheral blood lymphocytes (PBL) were cultured either alone or in co-cultures.

In co-cultures of AM with PBL, TMD did not induce any change in γ-interferon (γ-IFN) and interleukin-1 (IL-1) secretion, while it was able to increase the level of tumour necrosis factor (TNF) (TMD 100 μg·mL⁻¹, p<0.05 vs control cultures), and of granulocyte macrophage colony-stimulating factor (GM-CSF) (TMD 1, 10 and 100 μg·mL⁻¹ p<0.05, <0.01 and <0.01, respectively, vs control cultures). In cultures of AM alone, TMD did not induce changes in the levels of any of the tested cytokines, whilst in supernatants from PBL lymphocyte cultures, TMD at 1, 10 and 100 μg·mL⁻¹ increased the amounts of GM-CSF (p<0.01 each comparison vs control cultures).

Thus, TMD seems to directly stimulate lymphocytes to secrete GM-CSF and to modulate macrophage-lymphocyte interactions, resulting in the release of TNF and GM-CSF.


Thymomodulin is a calf thymus acid lysate composed of several thymic peptides with a molecular weight ranging from 1-10 Kda [1]. In vitro thymomodulin induces the maturation of T-lymphocytes, increases antibody production by B-lymphocytes and enhances the cytotoxic activity of natural killer (NK) cells [2–5]. In addition to lymphocytes, thymomodulin acts on other cell types involved in the inflammatory processes, such as phagocytes and their precursors [4, 6]. Because of these properties, thymomodulin has been used in patients thought to have defective inflammatory and immune mechanisms in the lower respiratory tract, such as individuals with chronic inflammatory airway diseases, or cancer patients treated with immunosuppressive therapy [7, 8]. However, no in vitro studies have been performed to evaluate the effects of thymomodulin on pulmonary mononuclear phagocytes, the alveolar macrophages. Most of the immune reactions in the lung in health and disease are based on the cellular interactions between alveolar macrophages and lymphocytes. Macrophages and lymphocytes communicate by means of a number of surface molecules and the release of a variety of cytokines able to induce the recruitment, activation and proliferation of inflammatory and immune effector cells [9–16].

With this background, the present study was designed to determine whether thymomodulin can modulate, in human alveolar macrophage and lymphocyte cultures, the release of various cytokines relevant to the inflammatory and immune reactions in the lung. Macrophages were obtained from the lower respiratory tract of individuals undergoing fibroptic bronchoscopy for clinical purposes, and cultured with or without autologus blood lymphocytes, and in the presence or absence of thymomodulin. The data show that thymomodulin acts on macrophages and lymphocytes inducing the release of increased amounts of tumour necrosis factor (TNF) and of granulocyte-macrophage colony-stimulating factor (GM-CSF).

Materials and methods

Study population

Seventeen individuals (8 males and 9 females, 28–60 yrs of age), referred to the First Division of Lung Diseases, San Martino Hospital, Genoa, Italy, entered this study. These individuals underwent fibroptic bronchoscopy as part of the evaluation for respiratory
symptoms, and/or for chest X-ray abnormalities, and were chosen according to the following criteria: 1) nonsmokers or former smokers who quit smoking at least 1 yr before; 2) taking no medication at the time of the study; 3) no known exposure to industrial or noxious pollutants; 4) lung function test results (total lung capacity (TLC), vital capacity (VC), forced expiratory volume in one second (FEV1), diffusing capacity of the lungs for carbon monoxide (DLco)) within normal values. The final diagnosis of these individuals was adenocarcinoma of the lung in 3 cases, small cell carcinoma of the lung in 2 cases, epidermoid carcinoma of the lung in 3 cases, localized metastatic lung involvement from tumours arising in other organs in 3 cases and tuberculosis in 1 case; whilst in 5 cases no evidence of any bronchial or lung disease was found. Five normal volunteers were used to obtain only peripheral blood lymphocytes to perform short-term cell cultures. Informed consent was obtained before venipuncture and bronchoalveolar lavage procedures.

Collection of lung and blood mononuclear cells

Cells were obtained from the lower respiratory tract of the 17 patients undergoing bronchoscopy through bronchoalveolar lavage (BAL), which was performed in all subjects with an Olympus BF-B2 bronchoscope (Olympus Corp. of America, New Hyde Park, NY, USA) using a total of 100 ml in five 20 ml aliquots of 0.9% sterile saline solution, as described previously [10]. When possible, to increase the number of cells recovered, two different lobes were lavaged (e.g. middle lobe and lingula) using a total amount of 200 ml of saline. To prevent blood contamination, BAL was always performed before biopsy and/or brushing and in areas free of any visible inflammatory or neoplastic endoscopic lesion or at the opposite site of the opacity visible on chest X-rays. The cells were separated by centrifugation (600g for 5 min), washed sequentially in Hank's balanced salt solution (HBSS) without Ca++ and Mg++, counted and resuspended at the desired cell density in the appropriate medium (see cell culture section). A small portion was taken for determination of viability; differential counts were performed on cytocentrifuge preparations stained with Diff Quik (Harleco, Dudingen, Switzerland).

Heparinized blood samples were obtained through venipuncture in the group of 17 patients, just before the fiberoptic bronchoscopy procedure, and in the five normal volunteers. Peripheral blood mononuclear cells were isolated by Ficoll density gradient [10], washed several times in HBSS without Ca++ and Mg++, resuspended in RPMI-1640 (Flow Laboratories, Irvine, Scotland, UK) with 10% autologous plasma and counted. To enrich for T-lymphocytes, blood mononuclear cell suspensions were layered onto culture dishes (Falcon culture dishes; Becton Dickinson, Sunnyvale, CA, USA) and allowed to adhere for a second 45 min at 37°C in 5% CO2. Non-adherent cells were then carefully removed from the dishes, washed twice in RPMI-1640, counted and allowed to adhere for a second 45 min period onto culture dishes. After removal and washing, the non-adherent cells were resuspended and counted; a small portion was taken for differential counts on cytocentrifuge preparations and for the assessment of the percentage of cells recognized as T-lymphocytes by monoclonal antibodies (Leu5, Becton Dickinson, anti-CD2 monoclonal antibody). The percentages of CD2+ T-cells in the final blood non-adherent cell suspensions, hereafter referred to as lymphocytes, were always >93%.

Thymomodulin

Thymomodulin (Ellem, Milan, Italy) is derived from calf thymus by acid lysis, filtration and lyophilization. The result is a mixture of peptides <10,000 Da in MW. Isoelectrofocusing assays have shown that thymomodulin consists of peptides distributed in the whole range of charge, although with more density in the acid (pH 3–6) part of the gel. In vitro and in vivo studies have shown that thymomodulin is able to regulate the maturation of human and murine pre T-lymphocytes, as well as to act on functional activities of mature human and animal B- and T-lymphocytes [1–5].

Cell cultures

This study included cultures of: 1) alveolar macrophages plus autologous peripheral blood lymphocytes; 2) alveolar macrophages; and 3) peripheral blood lymphocytes. Cells were cultured in two different media: a) for the determination of γ-interferon (γ-IFN), interleukin-1β (IL-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF) levels in culture supernatants, cultures were performed in RPMI-1640 supplemented with 5% heat inactivated foetal bovine serum (FBS) (Flow), glutamine 20 mM, 100 U·ml⁻¹ of penicillin, and 100 μg·ml⁻¹ streptomycin [11–13]; b) for the evaluation of tumour necrosis factor-α (TNF) amounts in culture supernatants, the cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories), supplemented with glutamine 20 mM, 100 U·ml⁻¹ of penicillin, 100 μg·ml⁻¹ streptomycin [12, 14].

Firstly, with the knowledge that lymphocytes are present in the lower respiratory tract and interact with macrophages in the defence mechanisms of the lung [10, 15] and that the major known effects of thymomodulin are on lymphocytes [1], we tested the effects of thymomodulin in an in vitro system including macrophages and autologous peripheral blood lymphocytes. In this series of experiments, 3x10⁵ alveolar macrophages, obtained from 9 individuals undergoing fibrooptic bronchoscopy (6 patients with bronchogenic carcinoma or with metastatic lung
tumour, and 3 individuals without evidence of neoplastic lung involvement) were cultured in the media described above with 1x10^6 autologous peripheral blood lymphocytes in 1 ml final volume in 24-well plates, at 37°C in 5% CO₂ for 24 h with or without thymomodulin at different concentrations (1, 10 and 100 μg·ml⁻¹). The macrophage to lymphocyte ratio 3:1 was chosen since it is similar to that observed in the lower respiratory tract [11, 17].

Secondly, to determine which cell type was releasing cytokines in the presence of thymomodulin, macrophages alone or lymphocytes alone were cultured in the presence or absence of thymomodulin. Therefore 3x10^6 alveolar macrophages obtained from 8 individuals undergoing fiberoptic bronchoscopy (5 patients with primary or metastatic lung neoplasms, and 3 individuals without neoplastic lung involvement) were cultured in the conditions described above. Similarly, 1x10^6 peripheral blood lymphocytes from five normal subjects were cultured in the conditions described above. After 24 h cultures, supernatants were collected, centrifuged and stored frozen.

**Evaluation of the production of different cytokines in alveolar macrophage and blood lymphocyte cultures**

The supernatants recovered from the different cell cultures were assayed for the presence of four cytokines, γ-IFN, IL-1, TNF and GM-CSF. γ-IFN levels were assessed using a radioimmunoassay (RIA) system from Centocor (Malvern, PA, USA) based on the reactivity of samples, standards and controls with a mouse monoclonal antibody specific for γ-IFN, labelled with I¹²⁵. Radioactivity was determined using a gamma scintillation counter (Beckman Analytical). IL-1β production was determined using an enzyme-linked immunosorbent assay (ELISA) system (Cistron, Pine Brook, NJ, USA), in which samples reacted first with a monoclonal antibody specific for human IL-1β, then with an IL-1 rabbit antiserum. Reactivity of the samples was then revealed by an enzymatic colour reaction and the colour intensity of each well was measured with a microtitration plate reader at 490 nm (SLT Labinstruments, Salzburg, Austria). The levels of TNF-α were measured using a sandwich enzyme immunoassay system (T-Cell Sciences, Cambridge, MA, USA) between an anti-TNF monoclonal antibody and an enzyme conjugated monoclonal antibody with neutralizing properties against TNF. The amounts of GM-CSF were determined by an enzyme amplified sensitivity immunoassay (EASIA) (Medgenix, Fleurus, Belgium), using several monoclonal antibodies directed against distinct epitopes of the GM-CSF. The microtitration plates of TNF and GM-CSF assays were read as described above. The RIA for γ-IFN and the ELISA for IL-1β, TNF-α and GM-CSF are reported to have no cross-reactivity with active or inactive interferons, lymphotoxin and denatured TNF and other colony stimulating factors and cytokines.

Finally, due to the limited number of alveolar macrophages recovered with bronchoalveolar lavage, not all of the macrophage assays were performed in each patient. The numbers of patients whose cells were used for each assay were as follows. Macrophages and blood lymphocytes from 9 individuals (6 patients with neoplastic lung involvement, 3 without neoplastic lung involvement) were used for macrophage-lymphocyte cultures: 5 patients (2 with cancer, 3 without cancer) for γ-IFN levels, 6 subjects (3 with cancer, 3 without cancer) for IL-1 levels, 6 patients (3 with cancer, 3 without cancer) for TNF amounts; and 6 individuals (3 with cancer, 3 without cancer) for GM-CSF levels. Macrophages from 8 patients (5 with neoplastic lung involvement, 3 without neoplastic lung involvement) were used for macrophage cultures: 6 patients (4 with cancer, 2 without cancer) for TNF amounts; and 6 individuals (3 with cancer, 3 without cancer) for GM-CSF levels. Blood lymphocyte culture supernatants from 5 normal subjects were assayed for TNF and GM-CSF levels.

**Statistical analysis**

All data are presented as mean±standard error of the mean; statistical analysis for multiple comparisons was made with the analysis of variance (ANOVA) test and the two-tailed Student's test. The mean values of various parameters were said to be significantly different when the probability of the differences of that magnitude, assuming the null hypothesis to be correct, fell below 5% (i.e. p<0.05).

**Results**

**Characterization of cell populations recovered by lavage**

Bronchoalveolar lavage was performed in all subjects without complication. Visualization of the bronchial tree in the lavaged areas demonstrated no evidence of inflammatory or neoplastic lesions. The recovered fluid was sterile in all cases. The total amount of fluid recovered for each 100 ml of saline infused was 66±5 ml, and the total cells recovered were 11.7±1.8x10⁶, comprised of 90±2% macrophages, 6±2% lymphocytes and 1.0±0.2% neutrophils or eosinophils. There were no significant differences in the amount of fluid recovered, total and differential cell counts between the 11 patients with neoplastic lung involvement (8 patients with primary lung tumours, 3 patients with metastatic lung tumours) and the 6 patients without neoplastic lung involvement (1 patient with tuberculoma, 5 individuals with no evidence of lung diseases) (table 1).
Production of γ-interferon (γ-IFN), interleukin-1β (IL-1), tumour necrosis factor-α (TNF), and granulocyte-macrophage-colony-stimulating factor (GM-CSF) in co-cultures of alveolar macrophages and autologous blood lymphocytes. Cytokine levels were measured in supernatants from cultures with or without different concentrations of thymomodulin. Alveolar macrophages and blood lymphocytes were obtained from nine patients undergoing fibroptic bronchoscopy. A) γ-IFN levels; and B) IL-1, TNF, and GM-CSF levels. CTR: control medium; TMD 1, 10 and 100: thymomodulin 1, 10 and 100 µg·ml⁻¹. *: p<0.05; #: p<0.01, vs unstimulated cell cultures.

Cytokine production in alveolar macrophage-blood lymphocyte cultures with or without thymomodulin

Since the cellular interaction is an important step in macrophage and lymphocyte activation, we cultured alveolar macrophages with autologous blood lymphocytes and with or without thymomodulin. The supernatants were tested for the presence of γ-IFN, IL-1, TNF and GM-CSF. Supernatants from unstimulated cultures showed the presence of small amounts of γ-IFN (110±11 Ux10⁳) which were not increased by the addition of thymomodulin at all the concentrations tested (p>0.5 vs untreated cultures) (fig. 1A).

Similarly, unstimulated alveolar macrophages released a limited amount (56±4 pg·ml⁻¹) of IL-1, which was not increased in cultures performed in the presence of thymomodulin (p>0.5 for each comparison vs unstimulated cells) (fig. 1B). Unstimulated macrophage-lymphocyte cultures contained 40±2 pg·ml⁻¹ of TNF. At 1 and 10 µg·ml⁻¹ thymomodulin did not induce significant changes in TNF levels. However, the addition of 100 µg·ml⁻¹ of thymomodulin to alveolar macrophage-blood non-adherent cell cultures was associated with increased levels of TNF measured in culture supernatants (161±8 pg·ml⁻¹, p<0.05 vs unstimulated cells or cells stimulated with 1 or 10 µg·ml⁻¹ of thymomodulin) (fig. 1B). Concerning the other cytokines, GM-CSF was spontaneously released in supernatants from control cultures (32±5 pg·ml⁻¹) (fig. 1B). The addition of thymomodulin induced a dose-dependent increase of the levels of GM-CSF. In this context, in macrophage-lymphocyte cultures with 1 µg·ml⁻¹ of thymomodulin, we measured 86±6 pg·ml⁻¹ of GM-CSF, in cultures with 10 µg·ml⁻¹ of thymomodulin, 117±12 pg·ml⁻¹ of GM-CSF were present (both p<0.05 vs unstimulated cells), and 401±15 pg·ml⁻¹ of GM-CSF were found in cultures with 100 µg·ml⁻¹ of thymomodulin (p<0.01 vs unstimulated cells) (fig. 1B). Thus, in macrophage-lymphocyte cultures thymomodulin induced an increase of TNF and of GM-CSF levels.

Cytokine production in supernatants from cultures of alveolar macrophages and of blood lymphocytes with and without thymomodulin

TNF and GM-CSF can be produced by monocytes/macrophages and by lymphocytes in response to a variety of stimuli. To evaluate which cell type was releasing TNF or GM-CSF in the presence of thymomodulin, we cultured alveolar macrophages from 8 patients and blood lymphocytes obtained from 5 normal volunteers, and measured the levels of TNF and of GM-CSF in culture supernatants.

Unstimulated alveolar macrophages cultured for 24 h released 33±4 pg·ml⁻¹ of TNF (fig. 2A). The addition of thymomodulin to the cell cultures was not followed by any increase of the TNF levels (p>0.5 for each comparison vs unstimulated cells (fig. 2A).
THYMODODULIN STIMULATION OF AM AND FBL

Table 2. - Amounts of tumour necrosis factor and granulocyte macrophage colony-stimulating factor in supernatants of cell cultures from patients with and without neoplastic lung involvement

<table>
<thead>
<tr>
<th>Patients with cancer (n=3)*</th>
<th>TNF</th>
<th>GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR</td>
<td>39±3</td>
<td>158±6</td>
</tr>
<tr>
<td>TMD100</td>
<td>35±6</td>
<td>389±13</td>
</tr>
<tr>
<td>Patients without cancer (n=3)*</td>
<td>TNF</td>
<td>GM-CSF</td>
</tr>
<tr>
<td>CTR</td>
<td>42±2</td>
<td>165±8</td>
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<tr>
<td>TMD100</td>
<td>30±5</td>
<td>413±16</td>
</tr>
</tbody>
</table>

*: see methods and results for diagnosis; p>0.2 each comparison between the two patient groups. TNF: tumour necrosis factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; TMD: thymomodulin; CTR: control.

Unstimulated cultures of blood lymphocytes contained 22±3 pg·ml⁻¹ of TNF. Thymomodulin added to the cultures at 1, 10 and 100 µg·ml⁻¹ did not induce any significant change of the levels of TNF (p>0.5 each vs unstimulated cells), (fig. 2C). Supernatants from unstimulated blood lymphocyte cultures contained 23±4 pg·ml⁻¹ of GM-CSF. The addition of thymomodulin to the blood lymphocyte cultures was associated with a dose-dependent increase of the amount of GM-CSF in the supernatants (thymomodulin 1 µg·ml⁻¹ 134±7 pg·ml⁻¹, thymomodulin 10 µg·ml⁻¹ 178±9 pg·ml⁻¹, thymomodulin 100 µg·ml⁻¹ 303±12 pg·ml⁻¹, p<0.01 each vs unstimulated T-lymphocytes), fig. 2D). Therefore, thymomodulin induced blood lymphocytes to release increased amounts of GM-CSF.

Finally, throughout the study, no significant difference was seen in the amounts of the measured cytokines between cultures of cells from cancer patients or from patients without evidence of neoplastic disease (p>0.2, each comparison). As an example, table 2 shows the levels of TNF and GM-CSF in cultures of macrophages plus autologous blood lymphocytes and in cultures of macrophages alone performed in control medium and in the presence of 100 µg·ml⁻¹ of thymomodulin.

Discussion

A number of thymic biological response modifiers have been proposed for patients with defective lung immune defence mechanisms. Although there is a great deal of data on the effects of these compounds on lymphocytes and thymic cells, little is known of their activities on alveolar macrophages and on the cellular interaction between macrophages and lymphocytes in the lung. We studied the in vitro effects of a calf thymus derivative, thymomodulin, on cultures of alveolar macrophages, of blood lymphocytes and on cocultures of macrophages and lymphocytes. The data demonstrate that thymomodulin induces an increase of the TNF and the GM-CSF levels in macrophage-lymphocyte cultures and an increase of the release of GM-CSF in lymphocyte cultures.

The presence of increased amounts of GM-CSF in supernatants from cells treated with thymomodulin is consistent with previous reports showing that thymus...
and thymic biological response modifiers play a role in the proliferation and differentiation of haemopoietic cells, basically by inducing other cells to secrete CSF [4, 6, 18, 19]. In contrast with GM-CSF, TNF levels were increased only in co-cultures of macrophages and lymphocytes with thymomodulin, and not in cultures of macrophages alone or of lymphocytes alone. In addition, the amounts of GM-CSF measured in blood lymphocyte cultures with or without thymomodulin were similar to the amounts of GM-CSF found in macrophage-lymphocyte cultures, suggesting that macrophages do not contribute significantly to the release of this cytokine. Taken together, these data suggest that, in macrophage-lymphocyte cultures, thymomodulin induces lymphocytes to release GM-CSF that subsequently stimulates the production of TNF, probably from macrophages. Consistent with this hypothesis is the demonstration that GM-CSF may stimulate the production of TNF [20]. Alternatively, cellular interactions between macrophages and lymphocytes might be needed for the secretion of TNF in the presence of thymomodulin, whilst they seem not necessary for the release of GM-CSF.

Although it is known that patients with lung cancer, may show in vitro abnormal cell activities, the data from this paper did not demonstrate any significant difference in cytokine production between cancer and non-cancer patients. Similar results were found when we evaluated ciliated bronchial epithelial cells for human leucocyte antigen DR (HLA-DR) expression and ability to stimulate in vitro, T-cell proliferation [21]. Nevertheless, we cannot exclude the possibility that in larger groups of patients, or in vivo, thymomodulin might exert different activities in cells from cancer patients than from patients without cancer.

The demonstration that thymomodulin induces cytokine secretion from macrophages and lymphocytes may suggest several considerations. It is known that cytokines possess pleiotropic effects. In this context, although originally described for its cytolytic activity on tumour cells, TNF is able to recruit, activate and modulate cell function of inflammatory and immunocompetent cells [13, 22, 23]. For instance, TNF enhances HLA-DR expression by monocytes/macrophages and increases the T-cell mediated responses to antigen stimulation [22-24]. Similarly, in addition to its growth factor activity on bone marrow precursor cells, GM-CSF recruits and activates different cell types, including macrophages, and increases the production of other cytokines, including TNF [13, 20, 25, 26]. Thus, thymomodulin might specifically increase the ability of resident lung cells to react in a variety of pathological conditions via the recruitment and activation of immunoeffectors cells.

In addition, the ability of thymomodulin to induce cytokine release from lymphocytes and from alveolar macrophages only in the presence of lymphocytes, suggests that thymomodulin acts primarily on lymphocytes, as expected from a thymic biological response modifier, but that this compound also influences the interactions between lymphocytes and macrophages in the lung. The in vitro biological activities of thymomodulin on macrophages and lymphocytes demonstrated in this study may explain, at least in part, the clinical effects of thymomodulin observed in patients with disorders associated with defective lung defence mechanisms.

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References


