Insulin-like growth factors and their binding proteins in pleural fluid

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Abstract

We investigated the expression and potential regulatory role of insulin-like growth factors (IGFs) and their specific binding proteins (IGFBPs) in tuberculous and nontuberculous pleuritis. By using a radio-immunoassay after acid gel filtration chromatography, we found that mean concentrations of IGF-I were 211.9 ± 20.2 µg/l and 203.2 ± 31.1 µg/l in pleural fluid of 14 patients with tuberculous pleuritis and 9 patients with malignant pleuritis respectively. These values were near those in serum of the same patients (221.3 ± 19.5 µg/l and 204.6 ± 21.0 µg/l respectively). By using a specific protein-binding assay, we found that mean concentrations of IGF-II were 345.3 ± 61.0 µg/l and 167.6 ± 22.7 µg/l in tuberculous and malignant pleural effusions respectively. These values were significantly lower than those in serum of the same patients (628.3 ± 79.0 µg/l, P<0.025 and 532.0 ± 85.9 µg/l, P<0.025 respectively). Because bioavailability and bioactivity of IGFs may be regulated by their binding to IGFBPs, we studied IGFBP patterns in the pleural fluid of 6 patients with tuberculous pleuritis. As assessed by Western ligand blotting the levels of IGFBP-1 and IGFBP-2 were increased whereas those of IGFBP-3 were decreased in pleural fluid in comparison with serum. The decrease in IGFBP-3 levels reflected increased proteolysis, as assessed by Western immunoblotting. In spite of this presence of IGFBPs, IGFs could be responsible for the local biosynthesis of 1,25-dihydroxyvitamin D (1,25-(OH)2D) since pleural fluid levels of both IGF-I and IGF-II significantly correlated with those of 1,25-(OH)2D. These results indicate that IGFs are detectable in pleural fluid and may contribute to control the activity of 25-hydroxyvitamin D-1α hydroxylase in tuberculous pleuritis.

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Introduction

Recent interest has focused on determining how the immune response against Mycobacterium tuberculosis is regulated (1). The finding that effective mechanisms restrict the growth of the bacilli in tuberculous pleuritis (2) has led to the study of inflammatory cells and inflammatory mediators or hormones particularly in the pleural space (3–8). The results of these investigations have indicated that accumulation of antigen-reactive T lymphocytes is associated with local production of 1,25-dihydroxyvitamin D (1,25-(OH)2D) (4). This may facilitate the elimination of mycobacteria by enhancing macrophage function while limiting tissue destruction by inhibiting lymphocyte proliferation. The mechanisms whereby the activity of 25-hydroxyvitamin D-1α hydroxylase (1αOHase) is locally up-regulated are still unknown. Most recent evidence indicates that regulation by interferon-γ (IFN-γ) may be a mechanism of control. Consistently, IFN-γ is concentrated at the site of tuberculous pleuritis (6–8) and, in vitro, IFN-γ stimulates 1,25-(OH)2D synthesis by macrophages (9, 10). Insulin-like growth factor-I (IGF-I), which has been shown to stimulate renal 1αOHase activity (11, 12), may also participate in the mechanism of control. In this regard, several investigators have reported that bone marrow-derived cells such as monocytes/macrophages and T cells, the predominant cells in tuberculous pleuritis, synthesize IGF-I (13, 14). However, IGF-I levels which have been determined in various fluids of the organism, including serum, lymph (15) and cerebrospinal fluid (16), were not assessed in pleural fluid.

In the present study, therefore, we examined the levels of IGF-I in pleural fluid of patients with tuberculous pleuritis as compared with malignant pleuritis. These investigations included evaluation of IGF-II and of IGF binding proteins (IGFBPs) that play a role in regulating the bioavailability of IGFs (17). In addition, to investigate whether 1αOHase activity is positively related to IGF bioavailability, 1,25-(OH)2D levels were subsequently examined.
Methods

Patient population

Pleural fluid and blood were obtained from 14 patients (aged 33±4 years) with tuberculous pleuritis examined at the Pulmonary Center of Tenon Hospital (Paris) and of Montfermeil Hospital (Montfermeil). Patients with antituberculous therapy and patients with positive serology for HIV were excluded. All these patients had unilateral exudative pleural effusions with or without radiological evidence for parenchymal infiltrates and cavitation. The diagnosis was confirmed in all the cases by pathologic demonstration of granulomatous lesions on pleural biopsy, and/or by evidence of Mycobacterium tuberculosis in the culture of pleural fluid or tissue. All the patients responded to antituberculosis therapy.

Pleural fluid and blood were also obtained from 9 patients (aged 60±6 years) with pleural effusions resulting from malignancies (extension of lung carcinoma to the pleura, 8 cases; pleural metastases from breast carcinoma, 1 case).

IGF-I, IGF-II, and IGFBP determinations

Samples of pleural fluid and peripheral blood were centrifuged at 2500 r.p.m. for 10 min and supernatants were harvested and stored at −70 °C. IGF levels were determined after separation by acidic gel (Ultrogel AcA54, Biosepra, Villeneuve La Garenne, France) chromatography using methods described previously (18). IGF-I was measured by radioimmunoassay (anti-IGF-I antibodies were kindly provided by Dr F Frankenne and Dr G Hennen, Centre Hospitalier de Liège, Belgium) and IGF-II by protein-binding assay using a cerebrospinal fluid binding protein with a selective affinity for IGF-II (19). IGF-I and IGF-II used both as standard and as tracer were generously provided by Ciba-Geigy Ltd (Basel, Switzerland).

IGFBP distribution patterns in serum and pleural fluid were studied by Western ligand blotting (20). Proteins in 3 µl samples of serum or pleural fluid were size-fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 15 h and then electroblotted onto a nitrocellulose sheet. Thereafter, this was treated with Nonidet P-40, BSA, and Tween-20 and incubated with 125I-IGF-I and 125I-IGF-II for 48–72 h at 4 °C. IGF binding proteins were detected by autoradiography (7–10 days exposure). Scanning densitometry of the autoradiograms was performed using a combined LKB Ultrosan laser densitometer and GelScan XL software (Pharmacia LKB Biotechnology, Inc., Uppsala, Sweden). The levels of IGFBP-3 were also measured by Western immunoblotting (21). The nitrocellulose sheets were washed and then incubated for 1 h at 37 °C with a 1:10 000 dilution of a polyclonal anti-human IGFBP-3 antibody. Bound antibody was detected using horseradish peroxidase-linked second antibody (Sigma, St Louis, MO, USA) and the blots were revealed using the ECL Western blotting detection system (Amersham International, Amersham, Bucks, UK). Scanning densitometry was performed as indicated above.

Vitamin D metabolite measurements

To measure 25-hydroxyvitamin D (25 OHD), 0.5 ml samples of serum or pleural fluid were extracted with a mixture of chloroform and methanol. The extract was chromatographed on a silicic column, and 25 OHD present in the purified samples was quantitated by a protein-binding assay, as previously described (22). The lower limit of detection was 0.2 ng/ml. To measure 1,25-(OH)2D, 1 ml samples of serum or pleural fluid were extracted with a mixture of methanol and methylene chloride. The extracts were chromatographed on a 1×20 cm column of Sephadex LH20 (Pharmacia Fine Chemicals, Piscataway, NY, USA) using an n-hexane/chloroform/methanol (90:10:10) solvent, and 1,25-(OH)2D present in the purified samples was quantitated using a radioreceptor assay, as previously described (22). The normal ranges for 1,25-(OH)2D and 25 OH D plasma concentrations were 20–50 pg/ml and 6–30 ng/ml respectively.

Statistical analysis

Values are presented as means±S.E.M. Differences between the results were evaluated by the Mann-Whitney U test or the Wilcoxon matched pairs test. Coefficients of correlation were determined according to the method of least squares.

Results

Measurement of IGF-I and IGF-II levels in pleural fluid

Differential cell counts in peripheral blood and pleural fluid showed that total cell numbers were significantly higher in tuberculous effusions compared with malignant pleural effusions, whereas percentages of neutrophils and lymphocytes and levels of proteins were not different (Table 1).

To quantify the accumulation of IGFs at the site of tissue inflammation, we first measured IGF-I concentrations in pleural fluid and serum in the two groups of patients (Fig. 1 and Table 2). Mean concentrations of IGF-I in serum from patients with tuberculous and nontuberculous pleuritis were comparable and slightly lower than those observed in serum from age-matched healthy subjects (Table 2). Compared with serum, pleural fluid concentrations of IGF-I were not different so that the mean pleural fluid/serum ratio was about 1.0 in the two groups of patients.
Next, IGF-II concentrations were assessed in the same samples (Fig. 2 and Table 2). In the two groups of patients, mean serum concentrations of IGF-II were moderately lower than those observed in healthy subjects. However, the mean IGF-II/IGF-I ratio was almost in the normal range (Table 2). By contrast with IGF-I levels, IGF-II levels were lower in pleural fluid than in serum, especially in tuberculous pleuritis.

**Potential regulatory role of IGF-I and IGF-II in tuberculous pleuritis**

Because IGFBPs modulate both the accumulation and the action of IGFs on their target cells, the electrophoretic profiles of IGFBPs were analyzed in pleural fluid and serum of six successive tuberculosis patients (Fig. 3A and Table 3). Western ligand analysis of serum showed four IGF binding species of ~42, 39, 34, 30 and 24 kDa. Bands at 42–39, 34 and 24 kDa represent IGFBP-3, IGFBP-2 and IGFBP-4 respectively, whereas the band at 30 kDa may represent IGFBP-1 and also glycosylated IGFBP-4, IGFBP-5, IGFBP-6 or fragments of IGFBP-3 (21, 23). Thus this band will be referred to as 30 kDa BP. Subsequent analysis by scanning densitometry indicated that IGFBP-3 was the major IGF carrier in serum from tuberculosis patients as in serum from healthy subjects (Table 3 and Fig. 3B). By comparison with serum, pleural fluid was characterized by a significant increase in the percentage of 30 kDa BP and IGFBP-2 and a significant decrease in that of IGFBP-3 and IGFBP-4. The mean pleural fluid/serum ratio for IGF binding capacity was 0.77 ± 0.29, 0.18 ± 0.04, 0.08 ± 0.02 and 0.01 ± 0.01 for 30 kDa BP, IGFBP-2, IGFBP-3 and IGFBP-4 respectively. Western immunoblotting using a specific anti-human IGFBP-3 antibody demonstrated specific bands at approximately 42, 39, 30, 18 and 16 kDa in serum of tuberculosis patients as in serum from healthy subjects (Fig. 3C). Bands at 42 and 39 kDa represent intact IGFBP-3 whereas bands at 30 kDa and 18/16 kDa correspond to the major and minor proteolytic fragments of IGFBP-3 respectively. In comparison with serum, pleural fluid was characterized by an increased proteolysis of IGFBP-3. Indeed, the approximately 30 kDa band accounted for 37.7 ± 5.5% and 67.6 ± 5.1% of the total immunoreactive IGFBP-3 in serum and pleural fluid respectively. Smaller fragments were also barely detectable.

To ascertain whether the accumulation of IGFs in pleural fluid from tuberculosis patients could be responsible for local stimulation of 1αOHase activity, concentrations of vitamin D metabolites were measured. Mean concentrations of 1,25-(OH)2D were 89.6 ± 14.5 pg/ml in pleural fluid and 44.1 ± 7.3 pg/ml in serum (Fig. 4A). Thus compared with serum, 1,25-(OH)2D levels were significantly increased (P < 0.05). Such a transpleural gradient of 1,25-(OH)2D, which was also observed in malignant pleural effusions (95.2 ± 22.7 pg/ml in pleural fluid vs 43.4 ± 7.6 pg/ml in serum, P = 0.05), was not associated with a transpleural gradient of 25 OHD. Indeed levels of 25 OHD were low in pleural fluid compared with serum (9.5 ± 4.6 vs 13.0 ± 7.3 ng/ml). There was a linear association between the concentration of 1,25-(OH)2D and that of either IGF-I (r² = 0.39, P < 0.05) or IGF-II (r² = 0.62, P < 0.01) in pleural fluid (Fig. 4B).

**Discussion**

The results of the present study indicate that IGF-I and IGF-II accumulate in pleural fluid and potentially
control 1eOHase activity in tuberculous pleuritis. Levels of IGF-I and IGF-II in serum of patients with tuberculosis as well as with malignancy are lower than those observed in the serum of healthy subjects. The IGF decrease occasionally observed in both groups of patients may be related to similar causes as the IGF-I decrease described in patients after surgical or infectious stress (24). Part of this decrease might be due to a reduction in IGF-I secretion because of alteration in nutritional status, and/or induction of resistance to the stimulatory action of growth hormone. Nutritional regulation of IGF-II expression is probably less marked (25).

Levels of IGF-I in pleural effusions of both tuberculous and nontuberculous etiology were in the same range as serum levels. These results contrast with those reported for IGF-I levels in various fluids besides serum. For instance, levels of IGF-I in vitreous fluid, cerebrospinal fluid, lymph and skin interstitial fluid are only 2% (26), 1% (16), 10–30% (15) and 18% (27) respectively of the levels in serum. The reason for these differences could be related to the capacity of inflammatory cells in pleuritis to synthesize IGF-I. Indeed, although hepatic production appears to account for the majority of circulating IGF-I, various cells are responsible for the production of IGF-I in the tissues. Interestingly, they include in particular bone marrow-derived cells such as monocytes/macrophages (14) and T cells, which are the predominant cells in tuberculous pleuritis. Furthermore, tumor necrosis factor α (TNFα) has been found to stimulate directly IGF-I expression in macrophages (28). Thus the enhanced expression of TNF in tuberculous pleuritis (7) may function to stimulate IGF-I synthesis by inflammatory cells. Alternatively, accumulation of IGF-II in the pleural fluid could reflect the fact that IGF-I is transported from the blood circulation, rather than synthesized locally. Such a mechanism is potentially amplified by the local expression of IGFBPs (mainly -1, -2 and -3) which bind IGF-II with high affinity (17), and prevent its back diffusion.

In contrast with IGF-I levels, IGF-II levels in pleural effusions of both tuberculous and nontuberculous etiology were low compared with serum levels. This was observed in spite of the local presence of IGFBPs with higher affinity for IGF-II than for IGF-I. Thus the IGF-II/IGF-I ratio was even lower in pleural fluid than in serum. These results contrast again with those previously reported for IGF-II/IGF-I ratio in other fluids, especially in cerebrospinal fluid where this ratio is four times that found in serum (16). Interestingly, IGF-II levels were significantly higher in tuberculous pleural effusions as compared with malignant pleural effusions, whereas concentrations of total protein (Table 1) and IGF-I (Fig. 1) were not different. These data suggest that IGF-II could be synthesized by inflammatory cells that are in greater numbers in tuberculous pleuritis. However, no significant correlation was observed between IGF-II levels and cell numbers in pleural fluid from tuberculosis patients.

IGF bioavailability may be modulated locally by their binding to IGFBPs. A distinguishing feature of pleural fluid in tuberculosis as compared with serum is an augmented percentage of both 30 kDa BP and IGFBP-2.

Table 2 IGF-I and IGF-II levels in serum and pleural fluid. The concentrations (mean ± S.E.M.) correspond to the amounts of IGF-I and IGF-II measured after acidic gel filtration; number of samples in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>IGF-I (μg/l)</th>
<th>IGF-II (μg/l)</th>
<th>IGF-II/IGF-I ratio</th>
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<tbody>
<tr>
<td>Tuberculous pleural effusions</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Serum</td>
<td>221.3 ± 19.5 (14)</td>
<td>628.3 ± 79.0 (11)</td>
<td>2.9 ± 0.3 (11)</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>211.9 ± 20.2 (14)</td>
<td>345.3 ± 61.0 (13)</td>
<td>1.7 ± 0.2 (13)</td>
</tr>
<tr>
<td>Pleural fluid/serum</td>
<td>1.0 ± 0.1 (14)</td>
<td>0.6 ± 0.1 (11)</td>
<td></td>
</tr>
<tr>
<td>Serum from age-matched healthy subjects</td>
<td>296 ± 8 (53)</td>
<td>1090 ± 62 (18)</td>
<td>3.5 ± 0.2 (12)</td>
</tr>
<tr>
<td>Malignant pleural effusions</td>
<td></td>
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<tr>
<td>Serum</td>
<td>204.6 ± 21.0 (9)</td>
<td>532.0 ± 85.9 (5)</td>
<td>2.8 ± 0.1 (5)</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>203.2 ± 31.1 (8)</td>
<td>167.6 ± 22.7 (5)</td>
<td>1.1 ± 0.2 (5)</td>
</tr>
<tr>
<td>Pleural fluid/serum</td>
<td>1.0 ± 0.1 (8)</td>
<td>0.3 ± 0.0 (5)</td>
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<tr>
<td>Serum from age-matched healthy subjects</td>
<td>209 ± 15 (13)</td>
<td>1090 ± 62 (18)</td>
<td>4.5 ± 0.2 (8)</td>
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</table>

Figure 2 Concentrations of IGF-II in serum (x) and pleural fluid (■) of tuberculosis and control patients.
and a reduced percentage of IGFBP-3 (Table 3 and Fig. 3B). The mechanisms whereby IGFBP levels are regulated in pleural fluid have not been determined in this study. However, the ability of serine proteases to promote a limited proteolysis of IGFBP-3 and, hence, to reduce its affinity for IGF in ligand blotting (29) suggests a role for IGFBP-3 degradation. This structural alteration of IGFBP-3 was confirmed by immunoblotting. In addition, IGFBP-3 diffusion from blood into pleural fluid could be limited as compared with that of IGFBP-1 and IGFBP-2 because of differences in molecular weight. Alternatively because TNF is capable of increasing IGFBP-1 levels in the circulation (24), we hypothesize that 30 kDa BP accumulation in pleural fluid could be promoted by the local production of this and other cytokines (7).

The function(s) of IGFs and IGFBPs in tuberculous pleuritis must remain speculative. IGF-I has been shown
to stimulate 1,25-(OH)₂D production from 25 OH D in cultured mammalian kidney cells by increasing 1αOHase activity (11, 12). Because we found higher 1,25-(OH)₂D concentrations in pleural fluid compared with serum and a linear association between pleural fluid levels of 1,25-(OH)₂D and IGF-I or -II, the possibility exists that local IGFs modulate 1αOHase activity in pleuritis cells as well. However, such a regulation would also depend on IFN-γ, as previously demonstrated in in vitro experiments with macrophages (9, 10). Ongoing studies will determine the respective role of IGFs and IFN-γ in 1,25-(OH)₂D synthesis. Finally, IGFs are potentially involved in the process of tissue repair and fibrosis. This role is suggested by the known capacity of IGF-I to induce fibroblast proliferation (30).

In summary, we first demonstrated the expression of IGF-I and to a lesser extent IGF-II in both tuberculous and malignant pleural effusions. We suggest that IGFs may locally contribute to inflammation, repair and the fibrosis process in tuberculous pleuritis.

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