Coagulation, angiogenesis and cancer
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Plasma cytokine and P-selectin plasma levels in advanced malignancy: prognostic value and impact of low-molecular-weight heparin administration

Marcello Di Nisio, Tatjana M. Niers, Pieter H. Reitsma, Harry R. Büller
Chapter 8

Abstract

Background
The survival benefit described in patients with cancer treated with low molecular weight heparin (LMWH) may result from a LMWH-mediated effect on the immune system or on the cross-talk between platelets and tumor cells.

Methods
Plasma levels of interleukin (IL)-10, IL-6, interferon (IFN)-γ, and P-selectin were measured in patients with advanced stage malignancy who were randomized to receive standard cancer care with or without the addition of LMWH.

Results
Patients with IL-6 levels above the median had a median survival of 6.5 months versus 8.8 months for those with values below this cutoff (P = 0.02). IL-10 levels were found to be similarly correlated with survival such that IL-10 concentrations above the detection limit of the assay were associated with a doubled risk of dying in comparison to undetectable IL-10 (P = 0.02). No significant association was found between survival and circulating levels of IFN-γ. For P-selectin, patients with values below the fourth quartile had a median survival of 8.8 months versus 6.5 months for patients with levels above the fourth quartile (P = 0.02). In multivariate analysis, IL-10 remained an independent unfavorable prognostic factor (hazard ratio, 2.13; 95% confidence interval, 1.08-4.20). In patients treated with LMWH, the plasma levels of IL-6, IL-10, IFN-γ, and P-selectin demonstrated similar correlations with survival. However, none of the markers was associated with the beneficial survival effects observed with the administration of LMWH.

Conclusion
IL-10, IL-6, and P-selectin levels predicted a poor outcome in patients with advanced stage malignancy. The prolongation in survival observed with LMWH in patients with cancer apparently cannot be explained by a LMWH effect on these circulating markers.
Cytokine en P-selectin plasma levels in cancer

Introduction

Inflammation and innate immunity are considered essential in the defense against cancer\(^1\)\(^-\)\(^4\). Several studies have suggested that host responses are often defective in patients with cancer favoring, rather than opposing, the progression of the tumor\(^1\),\(^2\). Both tumors and innate immunity cells can produce immunomodulating agents that divert the host-protective mechanisms and suppress tumoricidal activity leading to a predominant humoral immunity, ineffective against the tumor\(^1\)\(^-\)\(^4\).

Accumulating data suggest that the plasma levels of some cytokines might reflect the immune system activity against the tumor and correlate to the extent of disease and prognosis\(^5\)\(^-\)\(^19\). Interleukin (IL)-6, for example, can stimulate cell growth and angiogenesis and induce resistance to therapy in cancer cells\(^1\),\(^3\),\(^20\), and high serum IL-6 levels have been found to predict a poorer clinical outcome\(^5\)\(^-\)\(^8\),\(^10\)\(^-\)\(^16\).

In addition, animal studies have shown an impairment of the host response against the tumor due to an abnormal production of IL-10 by the malignant and host immune cells\(^21\),\(^22\). To our knowledge, the prognostic value of IL-10 levels in humans remains uncertain with conflicting data reported in the literature\(^6\),\(^17\),\(^19\),\(^23\). Similarly, the possible prognostic value of other cytokines, such as interferon (IFN)-\(\gamma\), that promote a cell-mediated immune response has not been clearly evaluated to the best of our knowledge\(^24\)\(^-\)\(^26\). Recently, a role for low-molecular-weight heparin (LMWH) in the management of cancer patients has been claimed by several clinical studies based on a prolongation of survival in patients with cancer who were treated with LMWH in addition to standard cancer care\(^27\)\(^-\)\(^32\). The beneficial effects of LMWH on survival could be related to an effect on the host immune response, although data are limited with discordant results across the studies, possibly due to differences in experimental conditions\(^33\)\(^-\)\(^38\). Another possible mechanism with which to explain the anticancer activity of LMWH could be that LMWH interferes with the the cross-talk between platelets and cancer cells. Platelet have the potential to promote several steps of the tumor progression and markers of platelet activation have been correlated to a worse prognosis in patients with cancer\(^39\)\(^-\)\(^41\). It has been suggested that LMWH can inhibit tumor metastasis by blocking P-selectin\(^42\), a marker of platelet activation that has been with mortality and recurrent disease risk in patients with malignancy\(^43\).

The aim of the current study was first, to evaluate whether the plasma levels of P-selectin, IL-6, IL-10, and IFN-\(\gamma\) predict survival in patients with advanced stage cancer. Second, we assessed whether the levels of these markers were correlated with the response to LMWH treatment.

Materials and Methods

Patients
Plasma samples were obtained from patients participating in the malignancy and low-molecular-weight therapy (MALT) trial\(^28\). In that study, 302 patients without signs
or symptoms of venous thromboembolism and with a diagnosis of advanced solid malignant tumor, that was not curable with the standard available treatment were randomized to receive 6-weeks cycle of subcutaneous nadroparin (Sanofi-Synthelabo, Paris, France) or placebo. The characteristics of the MALT patients have been described in more detail elsewhere. Briefly, patients with a life expectancy of <1 month, an indication for anticoagulant treatment, a contraindication for LMWH, thrombocytopenia (defined by <50,000 platelets/mm³) or who were pregnant were excluded from the study. At baseline, data were collected concerning the demographic characteristics, as well as information on the type, histology, stage and duration of cancer. Moreover, the World Health Organization (WHO) performance status and the physician's assessment of life expectancy (< 6 months vs. ≥ 6 months) were determined. Patients were followed until death or until the end of the study, with a median follow-up of 12 months. In the intention-to-treat analysis, treatment with nadroparin was associated with a significantly prolonged survival with the greatest effects noted in the subgroup of patients with a better prognosis at baseline. The overall hazard ratio (HR) for mortality was 0.75 (95% confidence interval [CI], 0.59 to 0.96) in favor of LMWH.

In the current analysis, IL-6, IL-10, IFN-γ, and P-selectin levels were determined in an unselected group of 141 patients from the MALT study for whom plasma samples were available. Of these patients, 75 were randomized to receive nadroparin.

**Study objectives**

We sought to evaluate: 1) the prognostic value for survival of circulating IL-6, IL-10, IFN-γ, and P-selectin in all the 14 patients at the time of entry into the study; 2) the association between these circulating markers and prognosis in the group of patients treated with LMWH; 3) whether the beneficial survival effects observed in the MALT study regarding survival were related to the influence of LMWH on plasma levels on soluble P-selectin or cytokines. This latter effect could be reflected by changes of immune mediators and plasma concentrations. Given the role of IL-6, IL-10, and IFN-γ in the host response and in the promotion of tumor progression, a increase in IFN-γ and IL-10 was hypothesized to result from the administration of LMWH.

**Blood sampling and sample analysis**

At the start of study-treatment (Time 0) and at 6 weeks (the end of LMWH-treatment phase) a blood sample was obtained, anticoagulated with sodium citrate (0.109 M, 1/10 volume/volume). Platelet-poor plasma samples were frozen in small aliquots and stored at –70°C until analysis. IL-6, IL-10, and IFN-γ levels were measured with the Bio-Plex Cytokine Assay (Bio-Rad, Veenendaal; The Netherlands). The detection range was 0.49 pg/ml - 32000 pg/ml. P-selectin plasma levels were measured by DuoSet enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Abingdon, United Kingdom) with a detection limit of P-selectin concentrations of 0.21 ng/ml.pg/ml.
Statistical analysis
The Chi-Square and the Mann-Whitney U tests were used for descriptive purposes. Survival estimates were calculated according to the Kaplan-Meier method with the analysis based on the time from randomization to death. Patients alive at the end of follow-up were censored. The Cox regression model was used to adjust for potential confounding variables i.e. life expectancy (< 6 months vs. ≥ 6 months), WHO performance status (≤1, 2, ≥ 3) concomitant treatment (chemotherapy, radiotherapy [RT], hormonal therapy or other antineoplastic treatment), type of cancer (breast, colorectal, cervical or other) and histology (adenocarcinoma, squamous carcinoma, squamous carcinoma, or other). Ninety-five percent CIs were calculated when appropriate. IL-6, IL-10, IFN-γ, and P-selectin demonstrate a non-normal distribution therefore median values were calculated. Because plasma IL-10 was detectable in only a fraction of patients, the predictive value of IL-10 was assessed with IL-10 as a dichotomous variable taking the value 0 when below the detection limit, or 1 otherwise. Finally, the association between IL-6, IL-10, IFN-γ, and P-selectin levels and prognosis as well as the effects of LMWH on these circulating markers (Mann-Whitney-Wilcoxon test) was evaluated in the group of patients treated with nadroparin. The statistical analysis was performed using the SPSS package for Windows, version 11.0 (SPSS Inc., Chicago, IL).

Results
The entire study group
The characteristics of the study population are detailed in Table 1. The median levels (range) at entry into the study for IL-6, IL-10, IFN-γ, and P-selectin were respectively 9.4 pg/ml (range, 0.6 to 438.8 pg/ml); 1.2 pg/ml (range, 0.6 to 24.1 pg/ml); 3.6 pg/ml (range, 2.0 to 322.2); and 4.3 ng/ml (0.7-11.9 ng/ml) (Table 1). The plasma levels of IL-6 predicted a shorter survival, with both the median and the quartiles of the IL-6 distribution dividing patients into groups with a significant difference in prognosis. The median survival for patients with IL-6 concentrations above the median was 6.5 months compared to 8.8 months for patients with IL-6 values below this cut-off (p=0.02). In other terms, the risk of dying was 56% higher in patients with IL-6 values above the median (HR=1.56; 95% CI, 1.05 to 2.30) (Figure 1). It is noteworthy that in the group of patients who were still alive at the end of the six-week study-treatment period (n=73), IL-6 maintained its prognostic value (8.0 months versus 11.0 months, respectively; p=0.007). The association of IL-6 with an adverse outcome was even more remarkable when the analysis included the extreme values of the IL-6 distribution. The median survivals in patients with IL-6 levels above and below the fourth quartile (24.3 pg/ml) were remarkably different (7.3 months and 13.4 months, respectively; p=0.018). Circulating IL-10 was similarly correlated with a poor prognosis. In patients in whom IL-10 was detectable, the median survival was lower than in patients with IL-10 values below the detection limit of the assay (3.3 months versus 8.2 months; p=0.02). This difference
Table 1. Main baseline characteristics of the study population (n=141). For variables with a normal distribution, values are presented as the mean and the standard deviation while for those without a normal distribution the median and the range are used. WHO=World Health Organization; IL=interleukin; IFN=interferon.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients (n=141)</th>
</tr>
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<tbody>
<tr>
<td>Age (at inclusion), years</td>
<td>62.3 (38.4-85.7)</td>
</tr>
<tr>
<td>Gender (Males/females)</td>
<td>83/58</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>73.2 (±11.5)</td>
</tr>
<tr>
<td>Months of follow-up</td>
<td>7.2 (0.6-51.1)</td>
</tr>
<tr>
<td>Months of cancer duration at baseline</td>
<td>16 (0-217)</td>
</tr>
<tr>
<td>Months of metastasis duration at baseline</td>
<td>5 (0-84)</td>
</tr>
<tr>
<td>WHO status (%)</td>
<td></td>
</tr>
<tr>
<td>0 or 1</td>
<td>90.8</td>
</tr>
<tr>
<td>2</td>
<td>7.1</td>
</tr>
<tr>
<td>3 or 4</td>
<td>2.1</td>
</tr>
<tr>
<td>Life Expectancy (%)</td>
<td></td>
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<tr>
<td>less than 6 months</td>
<td>48</td>
</tr>
<tr>
<td>at least 6 months</td>
<td>52</td>
</tr>
<tr>
<td>IL-6, pg/ml</td>
<td>9.4 (0.6-438.8)</td>
</tr>
<tr>
<td>IL-10, pg/ml</td>
<td>1.2 (0.6-24.1)</td>
</tr>
<tr>
<td>IFN-γ, pg/ml</td>
<td>3.6 (2.0-322.1)</td>
</tr>
<tr>
<td>P-selectin, ng/ml</td>
<td>4.3 (0.7-11.9)</td>
</tr>
</tbody>
</table>

Figure 1. Association between survival and IL-6 median for the whole study group at entry into the study. The vertical axis represents the cumulative survival (%) and the horizontal axis the follow-up (months). The dotted line shows survival of patients with IL-6 values above the median (9.4 pg/ml), the continuous line of patients with values above this level.
Figure 2. Association between survival and IL-10 as a dichotomous variable. The vertical axis represents the cumulative survival (%) and the horizontal axis the follow-up (months). IL-10 is defined detectable when within the detection limit of the assay, not detectable otherwise. (2A) Association between survival and IL-10 in the whole group. (2B) Association between survival and IL-10 in the nadroparin group. The dotted line shows survival of patients with detectable IL-10, continuous line of patients with not detectable IL-10.
corresponded to a risk of death two times higher in patients with measurable IL-10 (HR=2.14; 95% CI, 1.10 to 3.89) (Figure 2A). At the end of the study-treatment period, the number of patients with IL-10 within the range of the assay was too small to assess the IL-10 predictive value (n=5).

No association with survival was evident using the median IFN-γ levels as a cut-off point (p=0.37). It is noteworthy that prognosis tended to improve with increasing IFN-γ quartiles, and IFN-γ concentrations above the fourth quartile (5.1 pg/mL) were related to longer median survival (13.4 months) compared with lower levels (8.4 months) (p=0.78). The median survival was comparable between patients with P-selectin levels above or below the median (8.0 months versus 8.2 months; p=0.61). Significant differences in prognosis were evident at the more extremes of the P-selectin distribution. In particular, patients with P-selectin concentrations higher than the fourth quartile (5.4 ng/ml) had shorter survival (6.5 months) than those with lower values (8.8 months) (HR=1.72; 95% CI, 1.1 to 2.7). Such association with poor prognosis was, however, not found to be statistically significant at the end of the study treatment (8.0 months versus 10.1 months, respectively; p=0.46).

In univariate analysis, the study treatment (nadroparin or placebo), patient weight; a life expectancy ≥ 6 months; the treatment received during the study period (chemotherapy, surgery, radiotherapy or hormone treatment); and IL-10, IL-6, and P-selectin levels were all found to be significantly associated with survival. When adjusting in a Cox multivariate model for all possible confounders, IL-10 remained an independent prognostic marker (HR=2.12; 95% CI, 1.08 to 4.20). After regression analysis, the predictive value, the predictive value of both IL-6 and P-selectin levels was maintained, but was no longer no statistically significant (HR=1.44; [95% CI, 0.96 to 1.44] and HR=1.65; [95% CI, 0.99 to 2.73]).

Patients treated with LMWH

In the group of patients randomized to receive nadroparin, a similar inverse relationship with prognosis, as in the whole study group, was evident for IL-6, IL-10, and P-selectin at baseline. A shorter median was found with IL-6 above the median and the forth quartile in comparison to lower levels (10.1 months versus 6.4 months, p=0.10; and 3.3 months versus 8.8 months, p=0.04, respectively). In the same way, IL-6 concentrations at the end of the study-treatment period represented an unfavorable prognostic marker (p=0.001). Patients with a measurable IL-10 had poorer outcome than those in whom this cytokine was not detectable (median survival 3.0 months versus 8.8 months; p=0.0008) (Figure 2B). For both IFN-γ and P-selectin, there was no difference in prognosis relatively to any of the considered cut-offs. In multivariate analysis, IL-10 remained a predictor of poor prognosis (HR=10.8; 95% CI, 2.99 to 39.4). In contrast to what was expected, none of the circulating markers evaluated was affected by nadroparin and none correlated with the survival benefits found in the main study for the group randomized to LMWH. Surprisingly, an increase of IL-6 from 8.1 pg/ml at baseline to 10.4 pg/ml at the end of study-treatment
was observed in patients who received nadroparin (p=0.03). While IL-10 and P-selectin concentrations were basically unchanged after nadroparin administration, plasma IFN-γ levels raised as compared to baseline (1.7 pg/ml versus 3.6 pg/ml; p=0.48).

**Discussion**

Detectable IL-10 predicted shorter survival in patients with advanced malignancy and also after correction for other potentially confounding variables. The results are in keeping with previous data that suggested an involvement of IL-10 in the immune escape mechanisms of the tumor\(^{21,22}\) and a prognostics value of plasma IL-10 levels in patients with cancer\(^{8,17-19}\). The correlation between IL-10 and a worse outcome, however, has not been always consistent\(^{5}\). Differences in the spectrum of included cancers and/or in the disease severity likely explains these discrepant results. The size of the current study sample did not allow us to perform a subgroup analysis for cancer type. However, when correcting for tumor type and tumor histology in the multivariate analysis the relation between IL-10 and survival remained unchanged, suggesting a similar prognostic role for IL-10 across different types of cancer.

Circulating IL-6 has been found to be associated with an adverse outcome in a variety of tumors\(^{5,6,10,12-16}\). In agreement with the available literature, the current analysis found shorter survivals in those patients with high IL-6 levels (Figure 1). The progression of the malignancy could be promoted by IL-6 in several ways as for instance an induction of vascular endothelial growth factor release, the activation of the coagulation system, or a modulating effect on the immune system\(^{1-4,38,39}\).

The current study results demonstrate an association between poor prognosis and high levels of IL-10, an anti-inflammatory cytokine, as well as high values of IL-6, a pro-inflammatory marker. Therefore, these data appear to support the notion that despite the general activation of the immune system, host responses remain ineffective against the tumor and would indeed favor the progression of the disease\(^{1,2}\). IFN-γ represents an important marker of the “cell-oriented” immune response\(^{1,2}\). Experimental and preliminary clinical studies suggest that IFN-γ could reverse the defective immune response induced by other cytokines, such as IL-10, and favor the development of an effective response against the tumor\(^{24-26}\). To our knowledge, the current study is the first to evaluate the prognostic value for survival of circulating IFN-γ in patients with cancer. A non statistically significant prolongation in survival was found for patients with IFN-γ above the highest quartile. However, the relevance of this association needs to be evaluated further.

Several studies have suggested that platelets can promote tumor progression by regulating, for instance angiogenesis\(^{40}\) or favoring tumor metastasis\(^{41}\). Platelet activation could lead to the release of P-selectin, which, in turn, may facilitate the attachment of tumor cells to the vascular wall\(^{41}\). Indeed, plasma levels of P-selectin have been associated
to survival and disease recurrences in patients with cancer. The predictive role of P-selectin is supported by our findings (Figure 3).

In the current analysis, the influence of LMWH on the circulating levels of IL-6, IL-10, IFN-γ, and P-selectin also was assessed. Data from a growing number of clinical trials suggest that LMWH does improve the prognosis and prolongs the survival of cancer patients. Although the mechanisms behind the anticancer activity of LMWH remain poorly understood, these could involve an effect on the host immune response. However, this hypothesis was, not confirmed by the results of the current study, in which none of the measured cytokines was found to correlate with the beneficial effects of nadroparin. Although a change in these cytokine concentrations would have given an indication of the general immune system activity against the tumor, the lack of such effect cannot exclude a possible influence of LMWH on other immune markers or on immune pathways not reflected in circulating markers. In contrast to what was hypothesized initially, nadroparin treatment was associated with a modest increase in IL-6 levels, the clinical relevance of which to our knowledge remains unknown.

Figure 3. Association between survival and P-selectin for the whole study group at entry into the study. The vertical axis represents the cumulative survival (%) and the horizontal axis the follow-up (months). The dotted line shows survival of patients with P-selectin values above the forth quartile (5436.0 pg/ml), the continuous line of patients with values above this level.
The survival prolongation noted with LMWH could also be explained by an effect on pathways such as angiogenesis or on P-selectin-mediated interactions between platelets and tumor cells although the current data do not support this theory. Given the relatively small sample size, the results of the current study have to be interpreted with caution and mainly considered as hypothesis generating. Because the investigated group included a broad range of malignancies, it was not possible to determine a hypothetical cancer type-specific effect of LMWH. However, it is reasonable that a potential LMWH anticancer activity directed against general mechanisms of cancer progression, such as the immune system or on platelets, would be less dependent on the type of cancer.

Circulating IL-6, IL-10, and P-selectin appear to be predictive of an adverse prognosis in patients with advanced stage malignancy. Whether IL-10 circulating levels might help in guiding therapeutic decisions in patients with cancer remains to be evaluated. These markers were not sensible to the LMWH administration, leaving the question of how LMWH positively impacts cancer progression unanswered.

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