Pharmacokinetics and Immunological Aspects of a Phase Ib Study with Intratumoral Administration of Recombinant Human Interleukin-12 in Patients with Head and Neck Squamous Cell Carcinoma: A Decrease of T-bet in Peripheral Blood Mononuclear Cells

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ABSTRACT

The aim of this study was to evaluate the tolerability of intratumorally administered recombinant human interleukin-12 (rhIL-12) in patients with head and neck squamous cell carcinoma. Six patients were treated once a week at two dose levels of 100 or 300 ng/kg, respectively, up to 24 weeks. The primary end point was to assess the toxicity and safety of intratumoral injected rhIL-12 in head and neck squamous cell carcinoma patients; the pharmacokinetics and pharmacodynamics of rhIL-12 and any evidence of antitumor effect were also determined. Toxicity was mild, with prolonged grade 4 lymphopenia observed in only one patient. No dose-limiting toxicities occurred. In all six patients, the rhIL-12 was detectable in plasma within 30 min. Significant reductions in absolute number of peripheral blood lymphocytes and all lymphocyte subsets, especially cytotoxic T cells and natural killer cells, were observed that were maximal between 12 and 24 h. Maximal plasma concentrations of IFN-γ and IL-10 were detected after 12 h. A realtime semiquantitative PCR analysis in peripheral blood mononuclear cells showed a mean increase of mRNA encoding IFN-γ of 2.2 times relative to the pretreatment sample. An unexpected, significant decrease of 80% in T-bet mRNA, a T-helper 1 transcription factor, was detected after 12 h, with normalization after 48–72 h. No complete or partial responses were observed. In one patient, a 40% regression of a tumor lesion was noted. In conclusion, rhIL-12 at these dose levels and schedule was well tolerated and resulted in measurable immunological responses.

INTRODUCTION

HNSCC provides an excellent model to study the effects of locoregional immunotherapy such as cytokines. HNSCC is characterized by the occurrence of regional lymph node metastases, and therefore, a regional lymph node dissection, together with a resection of the primary tumor, is frequently the treatment of choice. Furthermore, HNSCC is an immunogenic tumor, as demonstrated by a variable amount of infiltrating lymphocytes and other immune cells susceptible for activation by e.g., IL-2 treatment (1). Several kinds of immunodysfunction, starting at the site of the tumor and eventually generalized, have been reported (2–5). Immunotherapy given preoperatively allows to study the effects both in the primary tumor and in the (draining and non-draining) lymph nodes. In ~50% of patients, the primary tumor is accessible for local injection.

In the past, the therapeutic effects of locoregional IL-2 administration in HNSCC have been studied extensively. Locoregional immunotherapy may be preferred over the systemic administration for the following reasons. In animal models (6–9), it has been shown that with locoregional IL-2 higher concentrations of IL-2 are achieved at the site of the tumor with less toxicity compared with systemic treatment. Furthermore, after local administration, IL-2 has preferential access to the draining lymph nodes. Mattijssen et al. (10) showed in a syngeneic guinea-pig line-10 tumor model that i.t. and not peripherally administered PEG-IL2 resulted in the cure of animals with palpable experimental tumors and regional lymph node micrometastasis. Moreover, protective systemic antitumor activity was induced (10). In a Phase II study in HNSCC patients, the effect of i.t. injection of PEG-IL-2 was evaluated. It was feasible, however, the objective response rate was only 6% (11).

IL-12 is a heterodimeric cytokine, which consists of two disulfide-linked subunits, i.e., IL-12p40 and IL-12p35 (12).
IL-12 has many biological activities (13, 14). It stimulates the proliferation and activation of cytotoxic T lymphocytes and NK cells and induces the production of a number of other cytokines, especially IFN-γ, but also tumor necrosis factor α and granulocyte macrophage colony-stimulating factor (15, 16). IL-12 is the key cytokine in the induction of Th1 responses and thereby cellular immunity (17). Furthermore, IL-12 inhibits angiogenesis (18, 19). In experimental murine tumor models, IL-12 has antineoplastic activity (20, 21). In cancer patients with various tumor types, six Phase I studies (22–27) and one Phase II study (28) have been performed with either i.v. or s.c. administration of rhIL-12. Toxicity was very dependent on the schedule of administration (28, 29). Some clinical responses were observed. However, IFN-γ production induced in vivo by rhIL-12 was attenuated rapidly with consecutive cycles, which indicates that the biological response to rhIL-12 is down-regulated during therapy (22–24).

The primary objective of this study was to assess the toxicity and safety of s.c. injected rhIL-12 at two dose levels in patients with recurrent HNSCC. Our treatment schedule was based on an earlier s.c. study in which biological activity was demonstrated at the two dose levels used (30). The secondary objectives were the assessment of clinical activity, the PK of rhIL-12 after i.t. administration, and the PD effects on leukocyte subsets and cytokine profiles in peripheral blood. We have also investigated the effect of rhIL-12 in vivo on the expression level of mRNA of T-bet and c-Maf. T-bet is a recently described Th1-specific transcription factor that controls the expression of IFN-γ (31). T-bet expression correlates well with IFN-γ expression in Th1 cells, NK cells, and B cells. It redirects effector Th2 and T-cytotoxic 2 cells into the Th1 and T-cytotoxic 1 pathway by inducing a striking increase in production of IFN-γ and a repressed production of IL-4, IL-5, and surprisingly also IL-2. IL-12 induces an increase in levels of T-bet RNA and protein in vitro (31), c-Maf is a Th2-specific transcription factor. It regulates the expression of the key Th2 cytokine IL-4 (32).

PATIENTS AND METHODS

Patient Selection. All patients had histological proof of recurrent HNSCC with either local recurrence or s.c. or lymph node metastases not amenable for curative surgery or radiotherapy. The tumor had to be accessible for local injections. WHO performance diameter not exceeding 5 cm. Additional eligibility criteria for resectability of the tumor had to be accessible for local injections with a metastases not amenable for curative surgery or radiotherapy. Patients were treated once weekly for a period of 8 weeks. After 8 weeks, patients were evaluated for response. Patients without disease progression or DLT could receive up to 24 injections of rhIL-12 i.t. The first injection was given as inpatient treatment. All of the remaining doses were administered on an outpatient basis, with an observation period of 1 h after each injection.

Toxicity was assessed using the National Cancer Institute Common Toxicity Criteria Version 1. DLTs were defined as drug-related grade 3 or 4 toxicity, with the exception of lymphopenia. If no DLT occurred in three patients at 100 μg/kg, the dose was escalated to 300 μg/kg. If one of three patients experienced a DLT, three more patients were added to this dose level, and dose escalation was only allowed if less than or equal to two of six patients experienced DLT at 100 μg/kg.

Before start of the study, all patients underwent a complete medical history, physical examination, electrocardiography, hematology and blood chemistry tests, urinalysis, occult blood test in stool, chest X-ray, and evaluations of disease parameters by computed tomography scan.

Patients were monitored by physical examination, complete blood count, and serum chemistry before each weekly injection. Disease parameters were evaluated after each treatment period of 8 weeks by computed tomography scan. WHO criteria for response were used.

PK and PD Sampling and Parameters. Venous blood was collected in week 1 and week 6. Whole blood samples anticoagulated in EDTA and citrate were obtained for determination of total and differential leukocyte counts, separation of PBMCs by Percoll density centrifugation and immunophenotypic analysis before and after 4, 12, 24, 48, 72, and 96 h. Plasma was obtained for a determination of serum cytokine levels before and after 0.5, 1, 2, 4, 8, 12, 24, 48, 72, and 96 h.

The total number of leukocytes and differential leukocyte counts were determined on the Advia 120 (Bayer). Plasma IL-12 p70 and IL-12p40 were measured in an ELISA as described previously (33). For measuring of IFN-γ and IL-10, commercial ELISA kits were used (Sanquin Reagents, Amsterdam, The Netherlands). They were standardized with the standards of the National Institute for Biological Standards and Control.

After Percoll density centrifugation, the PBMCs were incubated with monoclonal antibodies and stained indirectly with FITC and/or phycoerythrin. The following monoclonal antibodies (and clones) were used: CD3 (T3b); CD4 (RIV7); CD8 (WT82); CD20 (B9E9); and CD56 (NBL-1). Thereafter, the PBMCs were analyzed by flow cytometry using a FACScan of Becton Dickinson (Mountain View, CA).

Total RNA of PBMCs was extracted using Trizol Reagent (Life Technologies, Inc.). Reverse transcription was performed using 5 μg of total RNA, random hexamers, and Moloney murine leukemia virus reverse transcriptase (Life technologies, Inc.). Predeveloped assay reagents (PE Biosystems) were used for determination of IFN-γ, IL-10, IL-12p35, and IL-12 p40. A predeveloped assay reagent (PE Biosystems) was used for de-
termination of a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. Taqman probes for T-bet, c-maf, and PBGD, a housekeeping gene, were synthesized by PE Biosystems (Branchburg, New Jersey). The sequence of the primers and probes of T-bet, c-maf, and PBGD used in this study are given in Table 1. Real-time sequence quantitative analysis was performed as described before (34). At several time points, the amount of mRNA of cytokines or transcription factors was normalized to glyceraldehyde-3-phosphate dehydrogenase and calculated relative to the amount of target present before start of rhIL-12. A cycle time (CT) of <35 was established as meaningful.

RESULTS

Patient Characteristics

A total of six patients were entered in the study: five males and one female, with a median age of 57 years (range, 28–70 years). The median WHO performance was 1 (range, 0–2). Tumor sites were: larynx (n = 3); oral cavity (n = 1); nasopharynx (n = 1); and oropharynx (n = 1). All patients had prior surgery and local radiotherapy. None of the patients had received prior systemic treatment. The rhIL-12 was administered at the site of a local recurrence in two patients and in regional lymph node metastases in 4 patients. In one of these four patients, the rhIL-12 was injected in two lymph node metastases. The volume of the injected rhIL-12 varied between 0.26 and 0.64 ml, dependent of the dose level and the weight of the patient. In some patients, rhIL-12 was injected under ultrasound guidance. Three patients received 100 ng/kg and three patients 300 ng/kg i.t. The median number of injections/patient was 9 (range, 3–24).

Toxicity and Clinical Response

Toxicity was mild. Fever grade 2 was seen in all patients, generally occurring 8–12 h after the first injection. In all patients, fever was minimal or absent after subsequent injections, with the exception of one patient who had fever after each of the 24 injections. No DLTs were observed. Before start of the rhIL-12 injections, all patients had a lymphopenia with a mean number of lymphocytes of 697/ml (range, 600-1300/ml). At baseline, two of five patients had lymphopenia equivalent with a grade 2 and three patients with a grade 3 lymphopenia. After the first injection in four of five patients, a temporary lymphopenia grade 4 (<500/ml) was seen and in one patient a grade 3 (500–900/ml). In one patient (at 100 ng/kg), the grade 4 lymphopenia was prolonged and present most of the time during his 24 weeks of treatment. No infections were observed. No other grade 2, 3, or 4 toxicities were observed. Two patients experienced local itching at the site of the injection.

No partial or complete responses were seen. One patient received only three injections because of early progression. In two patients who were rapidly progressive before start of the rhIL-12 administration, a stabilization of the disease for 10 (at the 100 ng/kg) and 12 months (at the 300 ng/kg) was observed with a reduction in tumor size of 40% in one of these patients.

rhIL-12 Pharmacokinetics

Results of the pharmacokinetic studies are shown in Table 1. PK analysis after the initial injection was performed in five of six patients. One patient treated at 300 ng/kg was not analyzed as leakage of IL-12 from the injected tumor was seen probably because of ulceration and necrosis. In all patients, IL-12 was detected in plasma 30 min after rhIL-12 injection. In three of four patients, the area under the curve (AUC) and the maximal concentration (Cmax) increased in week 6 in comparison with the first injection. For all injections the mean t1/2 as measured in plasma was 7.2 h (range, 5.4–9.6 h).

Pharmacodynamics

Effects on Leukocyte Subsets. Before rhIL-12 treatment, most patients showed a low to normal absolute number of lymphocytes in the peripheral blood (Table 3). Significant reductions in absolute peripheral blood lymphocytes counts were seen within 4 h after administration of rhIL-12. The maximal
 subclasses were observed in week 22 (data not shown). The same reductions in all or lymphocyte subclass reductions were observed between the increase from 48 to 96 h and normalization at 168 h. A decrease was observed after 12 or 24 h, followed by a gradual decrease in most patients, but this decrease was less pronounced than for T-bet mRNA (Fig. 3). Therefore, we calculated a T-bet/Th2 transcription factor ratio by determining the T-bet/Th2 mRNA expression by qPCR.

**Effects on Plasma Cytokine Levels.** The effect of rhIL-12 on the plasma levels of IFN-γ and IL-10 was analyzed (Fig. 1). After 4–8 h, an increase of IFN-γ was seen in five of six patients with peak concentration occurring at 12 h. After 24 h, the level of IFN-γ decreased with normalization at 48 h. The mean maximal concentration was 65.1 pg/ml (range, n.d. to 158 pg/ml). No significant correlation was observed between the doses of IL-12 injected or the IL-12p70 plasma level and the amount of IFN-γ produced. In three of four patients, the IFN-γ production measured in the plasma was somewhat lower in week 6 compared with week 1.

IL-10 production followed the same pattern as for IFN-γ in each patient. An increase of IL-10 was first detected after 4–8 h and reached its maximum after 12 h (in one patient after 24 h). A decrease was seen after 24 h and normalization after 48 h. The mean maximal concentration was 27.4 pg/ml (range, n.d. to 63 pg/ml). Again, no correlation was observed between the two doses of rhIL-12 or the amount of IL-12p70 in plasma and the level of IL-10. There was no apparent correlation between the plasma levels of IL-10 and IFN-γ. In week 6, the IL-10 production was comparable with week 1.

**Effects on mRNA of Cytokines and Transcription Factors.** To determine the expression of mRNA of certain cytokines and two transcription factors, we performed a semiquantitative PCR analysis on five of six patients. In some cases, it was not possible to process the samples for reverse transcription-PCR because of the low number of PBMCs after 12 or 24 h.

Four hours after rhIL-12 administration, the amount of mRNA encoding IFN-γ present in PBMCs showed a mean increase of 2.2 times (range, 0.8–4.2) relative to the pretreatment sample. After 12 h, the IFN-γ mRNA expression was returned to baseline values (Fig. 2).

The mRNA encoding IL-10 did not increase, except in one patient who expressed the highest level of IFN-γ mRNA. In this patient, IL-10 mRNA was increased 2.8 times after 4 h to a maximum of 5.2 times after 72 h (Fig. 2).

The IL-12p35 mRNA showed an increase in three patients with a maximum after 12 or 24 h and decreasing after 48 h (Fig. 2). The expression of IL-12 p40 was too low for reliable measurements (data not shown).
c-Maf ratio. In all patients, this ratio decreased after IL-12 treatment with a mean decrease of 0.7 (Fig. 3).

**DISCUSSION**

We have studied various aspects of i.t. administration of rhIL-12 in HNSCC patients. A schedule of 100 or 300 ng/kg, given once weekly was well tolerated. No DLTs occurred. There are three explanations for the low toxicity. First, we administered a relatively low dose of rhIL-12 when compared with the maximum-tolerated dose of i.v. (500 ng/kg; Refs. 22, 23, 25, 27) or s.c. (1000 ng/kg; Refs. 24, 26) administration. Second, the route of administration of rhIL-12 was i.t. so locoregional administration. In studies using i.v. dose-levels of 100 and 300 ng/kg (22), much more grade 2 and 3 toxicities were seen. In two s.c. studies, no or some grade 2 or 3 toxicity was seen at this dose level (24, 27). Therefore, this i.t. route of administration is less toxic than the i.v. route and possibly less toxic than the s.c. route. Third, it is known that these patients do have a compromised immune system as is reflected in the low normal basal number of lymphocytes. This may result in a diminished secondary cytokine release, leading to decreased toxicity.

In this limited number of six HNSCC patients, we saw some clinical activity. In two patients, a long-lasting stable disease was observed, with a reduction in tumor size of 40% in one of these patients.

In all patients, IL-12 was already detectable in plasma 30 min after i.t. administration. This is in accordance with the observation of Mattijssen et al. (10) with PEG-IL-2. As shown in previous studies, the IL-12 measured in plasma was mainly rhIL-12, but the induction of IL-12 mediated through IFN-γ cannot be excluded. When we compare our PK data with i.v. (22) or s.c. (23, 24, 26) administration of rhIL-12, the AUC and Cmax is higher after i.v. and lower after s.c. administration, respectively, compared with i.t. administration. Our finding of an increment in AUC from week 1 to week 6 in three of four patients is in contrast with the results of Portielje et al. (26) who described a reduction in AUC after repeated s.c. administration. This may be explained by an intrinsic effect of IL-12 on the tumor vasculature. rhIL-12 is an inhibitor of tumor neoangiogenesis in mouse models (18). The effects of IL-12 on preexisting vessels in larger human tumors are less known. The increased AUC after i.t. administration in week 6 may possibly be because of a leakage of the rhIL-12 of the tumor into the vessels and circulation.

When the induction of IFN-γ and IL-10 is compared between the i.t. and i.v. or s.c. administration of rhIL-12, most (23, 25, 27), but not all (24) results, show higher IFN-γ peak concentrations and comparative IL-10 peak concentration (23, 27). After six injections of rhIL-12 i.t., the IFN-γ production appeared to diminish in contrast to the IL-10 production, which remained the same, suggesting different mechanisms of induction of IFN-γ and IL-10 after rhIL-12 administration.
The number of lymphocytes and lymphocyte subsets rapidly declined after rhIL-12 administration, and this was most prominent for NK and cytotoxic T cells. In some patients, only 10% of these cells was present in circulation after 12–24 h. This decrease of lymphocytes was the same in week 1 and week 6. In one patient, the effect was still present after 22 weeks. After rhIL-12 i.t. administration, the magnitude of comparable results have been reported for i.v. and s.c. administration of rhIL-12 (23, 25).

For the interpretation of our data on mRNA of cytokines and transcription factors, it is important to realize that rhIL-12 changes the percentage of Th cells, cytotoxic T cells, B cells, and NK cells over time. After 4 h, we measured a slight increase in IFN-γ mRNA. We found no differences in the mRNA of IL-10, except in one patient. This low expression may be explained by the fact that the highest induction of IFN-γ and/or IL-10 occurs in the regional lymph nodes and not in the circulation. Alternatively, the PBMCs in which expression of the mRNA of IFN-γ and/or IL-10 is induced may leave the circulation.

Our data on the concentration of IL-12 subunits showed more IL-12p35 expression after 12 h. The expression of IL-12p40 was below the detection limit, both before and after rhIL-12 treatment. The induction of IL-12p35, the rate-limiting subunit, may reflect a real increase in PBMCs, which produce more IL-12p35 after stimulation with rhIL-12 or the remaining cells in circulation, i.e., B-cells, have relative more expression of mRNA of IL-12p35.

Interestingly, a distinct and reproducible decrease in the mRNA level of T-bet was detected after rhIL-12 i.t. administration in each patient, both in week 1 and in week 6. This effect was maximal at 12–24 h and coincided with the decrease in total lymphocytes. c-Maf showed a similar decrease over time, although much less pronounced compared with T-bet. The T-bet/c-Maf ratio, which may be indicative for the Th1/Th2 balance, showed a decrease in all patients. This apparent contradiction may be explained that we may have missed a possible peak in T-bet gene expression occurring before our first sampling time. The maximal increase of IFN-γ mRNA was detected after 4 h and of plasma IFN-γ after 12 h. Thus, an up-regulation of T-bet mRNA of IFN-γ was seen after rhIL-12 i.t. administration. That study will provide additional insight in the effects of rhIL-12 on immunological parameters in the primary tumor and the regional lymph nodes.

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REFERENCES


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