Interleukin-12 has no Effect on Vascular Density, Perfusion, Hypoxia and Proliferation of an Implanted Human Squamous Cell Carcinoma Xenograft Tumour Despite Up-regulation of ICAM-1

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Abstract. Background: Interleukin-12 is an anti-angiogenic and antitumor agent in many transplanted murine tumour models. In a previous clinical study in head and neck squamous cell carcinoma patients treated with rhIL-12 the tumour turned pale, after an initial reddening. The aim of this study was to investigate the effects of rmIL-12 on the vasculature, blood perfusion, hypoxia and proliferation of tumour cells in an implanted human head and neck squamous cell carcinoma xenograft tumour, with a relatively large diameter, in Balb/c nu/nu mice over time. Materials and Methods: Established human squamous cell carcinoma xenograft tumours were intratumorally injected for 3 days with either 200 ng rmIL-12 or PBA. Mice were sacrificed at 4 different time points (between 8 hours and 8 days after the last injection), after administration of Pimonidazole, BrdUrd and Hoechst 33342. The tumour sections were quantitatively analysed with a semi-automatic method based on a computerised digital image analysis system, after immunohistochemical staining. Results: Despite a faster and higher up-regulation of anti-mouse ICAM-1 in the IL-12-treated tumours, no significant differences in vascular density, perfusion fraction, hypoxic fraction and BrdUrd labelling index were detected between IL-12-treated tumour and control tumours. Conclusion: We suggest that the main reason why the observation made in humans could not be confirmed in this mice study is the combination of a lack of an intact immune system in the Balb/c nu/nu mice and a relatively large tumour with probably a lot of mature vessels.

Interleukin-12 (IL-12) has antineoplastic and antimetastatic activity in many experimental mouse tumour models (1, 2). This can be explained by a wide range of biological activities of IL-12 (3, 4). IL-12 stimulates the proliferation and activation of cytotoxic T lymphocytes and NK cells and induces the production of several cytokines, such as IFN-γ, TNF-α and GM-CSF (5, 6). IL-12 is the key cytokine in the induction of T helper 1 responses and thereby in cellular immunity (7). Furthermore, IL-12 inhibits angiogenesis (8, 9), which is mediated by the CXC chemokines, interferon-inducible protein-10 (IP-10) and monokine induced by interferon-γ (MIG), both up-regulated by IFN-γ (9-12).

Most studies concerning the effect of IL-12 on inhibition of angiogenesis are performed in small tumours. The in vivo assays used include the mouse corneal angiogenesis assay (8), sponge implantation (13), a matrigel assay (9, 14-17), an intradermal tumour suspension assay (18), and the inoculation of tumour cells subcutaneously in a skinfold chamber (19). In some studies the vascular effects of IL-12 in larger tumours were examined by measuring vessel density (12, 20, 21) or by analysis of tumour blood flow with Doppler ultrasound (22). All the mentioned studies showed inhibition of neovascularization after IL-12 treatment.

Recently, in a phase Ib study, we treated six patients with recurrent or metastatic head and neck squamous cell carcinoma, with relatively large tumour volumes (diameter between 1 and 5 centimetres), with recombinant human IL-12 (rhIL-12) (23). RhIL-12 was administered intratumorally once a week. In four out of five evaluable patients the colour of the tumour changed during the course of treatment. Initially, a reddening of the tumour was seen.
After 3 to 5 weeks, the tumour developed a paler appearance, suggesting a vascular effect.

In view of this observation, the aim of the present study was to investigate the microenvironmental effects of recombinant mouse IL-12 (rmIL-12) intratumorally in an implanted human tumour model. Our hypothesis was that, after the start of treatment with IL-12, first an increase in perfusion occurs, allowing the accumulation of immune cells (e.g. natural killer (NK) cells) in the tumour, followed by the inhibition of angiogenesis and effects on existing vessels, which eventually may lead to tumour shrinkage. To test this hypothesis, the effects of rmIL-12 intratumorally (i.t.) administered on the vasculature, blood perfusion, hypoxia and proliferation in a human head and neck squamous cell carcinoma xenograft in nude mice were analysed over time.

**Materials and Methods**

**Mice, tumours and treatment with mouse rIL-12.** Five to six-week-old female Balb/c nu/nu mice, maintained in a specific-pathogen-free unit in accordance with institutional guidelines, were used in the experiments. The tumour line SCCNij3, derived from a moderately- to well-differentiated human squamous cell carcinoma from the larynx, was used. Tumours were passaged when they reached a diameter of 1.0 cm. Viable 1 mm³ tumour pieces were implanted subcutaneously in the right hind leg of the mice.

When the tumours reached a diameter of 6-8 mm the treatment with mouse rIL-12 (R&D Systems, Minneapolis, USA) was started. During 3 consecutive days (days 1, 2, 3) half of the tumours (n=25) were injected with 200 ng rmIL-12, diluted in 20 µl sterile phosphate-buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA), i.t. Control tumours (n=25) were injected with 20 µl PBS/BSA 0.1 % i.t.

Approval of the local ethical committee for animal use was obtained for these experiments.

**Administration of markers of hypoxia, proliferation, and perfusion and time schedule.** As a marker of hypoxia, pimonidazole hydrochloride (1-[(2-hydroxy-3-piperidinyl) propyl]-2-nitroimidazole hydrochloride (Natural Pharmaceuticals, International Inc., Belmont, MA, USA) was used (24, 25). Pimonidazole hydrochloride was administered intravenously (i.v.) at a dose of 80 mg/kg in a volume of 0.1 ml saline. The S-phase marker bromodeoxyuridine (BrdUrd) (Sigma, St Louis, MO, USA) was given intraperitoneally at 500 mg/kg in a volume of 0.5 ml saline. As a marker of tumour blood perfusion the fluorescent dye Hoechst 33342 (Sigma), dissolved in saline, was given i.v. at a dose of 80 mg/kg in a volume of 0.1 ml saline. Pimonidazole was injected 60 min, BrdUrd 15 min and Hoechst 33342 1 min before killing the animals.

The mice were sacrificed at 4 different time points: on day 3 (8 hours after the last administration of mouse rIL-12), on day 4, on day 8 and day 11. After weighing, tumour excision specimens were immediately stored in liquid nitrogen.

**Immunohistochemistry.** Complete tumour sections of 5 µm thickness were cut and stored at -80°C until stained. From each tumour, one tissue section was stained and analysed for perfusion, hypoxia and endothelial structures. A consecutive tissue section was stained and analysed for perfusion, proliferation and all nuclei. A third tumour section was stained with hematoxylin and eosin (H&E) to distinguish viable from necrotic and non-tumour tissue. Finally, in 7 IL-12-treated and 7 control tumours a fourth tumour section was used for ICAM-1 expression.

**Figure 1.** The mean weights (and 2x SEM) in IL-12-treated (●) and control (○) mice for the four time intervals.

A third tumour section was stained with hematoxylin and eosin (H&E) to distinguish viable from necrotic and non-tumour tissue. Finally, in 7 IL-12-treated and 7 control tumours a fourth tumour section was used for ICAM-1 expression.

**Endothelial structures and pimonidazole.** Prior to the immunohistochemical staining procedures, non-specific binding of the mouse antibodies was prevented by blocking with the M.O.M-kit™ (Vector Laboratories, Inc.) overnight at 4°C. Monoclonal liquid diluent (MLD, Euro-DPC, Breda, The Netherlands) pre-incubation was performed for 60 min at room temperature. The tissue sections were incubated for 45 min at 37°C with rabbit anti-pimonidazole antiserum (24) diluted 1:200 in undiluted 9F1 rat monoclonal to mouse endothelium (Department of Pathology, UMC Nijmegen, Nijmegen, The Netherlands) (26). The sections were then incubated for 45 min at 37°C with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rat antibody (Fab2) 1:200 (Jackson Immuno Research Laboratories, West Grove, PA, USA) and Cyanine2-conjugated donkey anti-rabbit antibody 1:200 (Jackson Immuno Research Laboratories, West Grove, PA, USA). Next, the tissue sections were rinsed and mounted with Fluorostab (ICN Pharmaceuticals, Zoetermeer, The Netherlands). BrdUrd. The DNA in the tissue sections was denatured with 0.2N hydrochloric acid for 10 min. To neutralise the pH, the sections were immediately rinsed in 0.1 M Borax for 10 min. M.O.M. blocking was performed overnight at 4°C and MLD pre-incubation...
was performed for 60 min at room temperature. Then the sections were incubated at 37°C for 45 min with Br-3, a mouse monoclonal to BrdUrd 1:25, (Caltag Laboratories, San Francisco, CA, USA). Next, the sections were incubated for 45 min at 37°C with TRITC-conjugated goat anti-mouse 1:200 (Jackson) followed by a 45-min incubation at 37°C with donkey anti-goat F(ab')2 TRITC-conjugate 1:200 (Jackson). All nuclei were stained with fast blue 1:1000 in PBS for 10 min. Tissue sections were coverslipped.

ICAM-1. After fixation with dry acetone, the sections were air-dried and pre-incubated for 20 min with PBS/0.5% bovine serum albumin (BSA), after which incubation with mAb YN1/1.7 (anti-ICAM-1, IgG2b; 2 μg/ml, 60 min, 37°C) followed (27). After washing 2 times in PBS, sections were incubated with biotinylated mouse-anti-rat (MRK-1, Pharmingen, San Diego, CA, USA, 5 μg/ml, 60 min, RT) in 1% normal mouse serum. Subsequently, PBS-washed sections were incubated with avidine-biotine peroxidase complex prepared as described by the manufacturer (Vector Laboratories Inc, Burlingame, CA, USA) for 45 min at RT. After rinsing twice with PBS, peroxidase activity was revealed using 3-amino-9-ethylcarbazole (AEC, substrate kit, Zymed, San Francisco, CA, USA) as substrate resulting in a red colour. After washing in water, cells were counterstained with hematoxylin. This method results in a red staining of cells expressing the ICAM-1 molecule.

Analysis of immunohistochemical markers. The tumour sections were quantitatively analysed with a semi-automatic method based on a computerised digital image analysis system, as described before. The hypoxic fraction (HF) was calculated as the proportion of the tumour surface stained for the hypoxic marker relative to the total tumour surface (28). Vascular density (VD) was measured as the number of vascular structures per mm² (29). The perfused fraction (PF) was calculated as the area of vascular structures labelled with the perfusion marker Hoechst 33342 divided by the total vascular area (30). The S-phase labelling index (LI) was calculated as the ratio of the BrdUrd-labelled area to the total nuclear surface (28).

The expression of ICAM-1 on the endothelial cells and the stroma in the tumours and the percentage of necrosis in the tumour was assessed qualitatively.

Statistical analysis. Analysis of variance (ANOVA) was used to assess the differences between the control mice and the IL-12-treated mice, and to assess the differences of PF, VD, HF and LI and at different times.

Prior to using ANOVA for each of the 8 groups of mice, the Kolmogorov-Smirnov test with Lillifors significance correction and Shapiro-Wilk tests were used as tests for normality, whereas the Levene statistic was used to test the homogeneity of variance. Pearson’s correlation analysis and linear regression analysis were used to disclose associations between the variables PF, VD, HF and LI in both control mice and the IL-12-treated mice.

All statistical analyses were performed with SPSS 10.0 for Windows.

Results

Growth, weight and necrosis. In 51 Balb/c nu/nu mice a human squamous cell carcinoma tumour was implanted on the hind leg. Twice a week the tumour diameter was measured and the tumour volume was calculated. After 26
days, the size of the tumours ranged from 6 to 8 mm and treatment with rmIL-12 i.t. was started in 25 mice and PBS/BSA 0.1% i.t. was administered in 25 control mice; one mouse without tumour growth was excluded.

The mean weights of the tumours and their 95% confidence interval for the IL-12-treated and control animals for the four time points (day 3 (8 hours after the last administration of rmIL-12), day 4, day 8 and day 11) are given in Figure 1. Analysis of variance disclosed that the differences of tumour weight at different times were not significant \((p=0.05)\). Contrary to expectation, the differences of the weight between the IL-12-treated and control mice for the four time points were not significant \((p=0.49)\).

The percentage of necrosis estimated on a H/E section ranged between 0% and maximal 15%. The median and mean necrosis was 0 and 1% in the control mice and 0 and 2% in the IL-12-treated mice, respectively, and did not differ between treated and untreated tumours.

**ICAM-1 staining.** Anti-mouse ICAM-1 expression was determined in 7 IL-12-treated and 7 control tumours. The IL-12-treated tumours showed a faster and higher expression, both in the endothelial cells and stroma, compared with the control tumours. After 8 hours, the ICAM-1 expression in the stroma was for 90% positive in the IL-12-treated tumours and only 10% in the control tumours.
tumours. So, this implicates the bioactivity of the administered rmIL-12. However, the ICAM-1 expression in the IL-12-treated tumours decreased after 4 and 7 days in contrast to the control tumours, in which a mild increase of ICAM-1 expression was seen in this time period.  

Vascular structures, perfusion fraction, hypoxia and proliferation. Figure 2 shows a detail of a combined composite image obtained after the scanning procedure of a representative tumour section in a control tumour. Figure 2A shows the perfused vessels (Hoechst), the vessels (9F1) and hypoxic areas (pimonidazole) distribution (100X magnification). This shows the relation between the vessels, partly perfused (pink) and the hypoxic areas in the tumour (green). The combination image of Hoechst, 9F1 and BrdUrd (Figure 2B) shows the spatial distribution of proliferating cells throughout the tissue section in relation to perfused and non-perfused vessels (200X magnification).

The perfused fraction, vascular density, hypoxic fraction and BrdU labelling index for the control and IL-12-treated mice for each of the four time intervals are given in Figure 3. ANOVA disclosed no significant differences between control mice and IL-12-treated mice for perfusion fraction, vessel density, hypoxic fraction and labelling index ($p \geq 0.27$).

An inverse relation was found between the number of vessels and the hypoxic fraction (Figure 4), both in the IL-12-treated (Pearson correlation $-0.553, p<0.01$) and the control mice (Pearson correlation $-0.845, p<0.01$). The difference in correlation between IL-12-treated and control mice was not significant ($p=0.6$).

Discussion
In this paper, we described the analysis of the microenvironmental effects of mouse rIL-12 i.t. in a human head and neck squamous cell carcinoma xenograft tumour line over time. Our hypothesis that IL-12 first induces an increase in perfusion, allowing the accumulation of immune cells (e.g., NK cells) in the tumour, followed by an inhibition of angiogenesis or changes in existing vessels, which eventually leads to tumour shrinkage, could not be confirmed. No significant changes in the vascular density, the tumour perfusion status, the hypoxic fraction or the proliferation status between IL-12-treated and control tumours in a time period ranging from 8 hours to 11 days post-treatment were found in this human tumour model. Neither a significant difference in the weight nor the percentage of necrosis between the IL-12-treated tumours and the control tumours were detected at any time point.

As expected a highly significant negative correlation between the vascular density and the hypoxic fraction was found. This correlation was stronger in the control tumours than in the IL-12-treated tumours.

A number of reasons for the lack of effect of the mIL-12 on the vasculature, perfusion, hypoxia and proliferation in this tumour mouse model can be suggested. First, the inhibition of angiogenesis by IL-12 is, largely, an immunological process, that is originated by induction of IFN-$\gamma$ and increased expression of IP-10 and MIG (9). Therefore, one might argue that the absence of a change in the micro-environment is caused by choosing BALB/c nu/nu mice in our experiments, lacking T cell functions and displaying enhanced NK activity (31). However, Strasly et al. (32) showed in BALB/c nu/nu mice, clear inhibition of angiogenesis after mIL-12 treatment, although impaired by 50% compared to normal mice. Also others (11, 33) have described inhibition of angiogenesis in BALB/c nu/nu mice in experiments with IL-12.

Second, we used a relatively short time schedule of IL-12 treatment by sacrificing the mice after maximally 11 days, i.e. 8 days after the last injection. This short time schedule was chosen to prevent the development of substantial necrosis in the tumour, which would complicate analysis of tissue sections due to an increase in artefacts resulting from non-specific staining. Indeed, the necrotic fraction was low in most tumours. However, Ogawa et al. found a clear inhibition of angiogenesis even with a short time schedule for injection (3 days) and with a short follow-up period (5 to 15 days) (34).

Third, the size of the tumour may be a factor in the absence of effect. We started the rmIL-12 treatment when the tumour was relatively large (6-8 mm in diameter).

Figure 4. Pearson’s correlation analysis disclosed a highly significant negative correlation between the vascular density and hypoxic fraction ($r=-0.71, p<0.001$) for the whole group of 50 mice. In the IL-12-treated (••••) and control mice (□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□
However, it is unlikely that the size of the tumour is the reason for the absence of an effect on tumour vasculature. Cavallo et al. (20) started treatment when the tumours were 2.5 mm in diameter, and Ogawa (34) when the tumour was 7 mm in diameter, both showing inhibition of angiogenesis.

Fourth, the micro-environmental characteristics of the tumour are important for the anti-angiogenesis effects of mrIL-12 in the tumour. Inhibition of angiogenesis by IL-12 was shown to exist in many tumour models, but not all. Factors indicated as critical for the inhibition of angiogenesis by IL-12 in tumours are the peritumoural stromal reaction (34, 35) and the lymphocyte-endothelial cell cross-talk (32). Therefore, we investigated the ICAM-1 expression in the tumour model used. A fast and high induction of ICAM-1 was detected in the IL-12-treated tumours, which decreased after 5 to 8 days after the last IL-12 injection. However, in the control tumours, which received an intratumoral injection of PBA, also a mild induction was seen, although it was clearly lower and later than in the IL-12-treated tumours. Thus, a reason for not detecting differences between the IL-12-treated and control tumours might be that, after intratumoral injection of PBA, an up-regulation of ICAM-1 is seen, also. However, if this were a main factor, one would expect to see time-related differences in any of the studied parameters in both groups.

The last reason for the lack of effect in our model, could be the maturation of the tumour vasculature. The level of vessel maturation is strongly correlated with the response to IL-12 treatment: mature pericyte-positive vessels were less sensitive (36). In our model we implanted viable 1 mm³ tumour pieces. In studies of IL-12 examining the effect of anti-angiogenesis, single cell suspension tumour cells were injected s.c. (15, 17, 34, 35, 37). Analysis of tumour at a smaller size may imply that a large proportion of vessels are immature relative to larger tumours, such as we used in our tumour model.

In conclusion, in the patients treated in the phase I study with rhIL-12 i.t. the observation in change of the colour of the tumour was very clear. However, in the described implanted xenograft tumour mice model, which was chosen because this mimics more closely the human situation than a model which injects single cell suspension tumour cells, we could not detect changes in vascular density, tumour perfusion status, hypoxic fraction or proliferation status, although an up-regulation of ICAM-1 was seen. Both in the human and mice study, we treated large tumours with probably a lot of mature vessels. The main difference between our human and mice study in Balb/c nu/nu mice is the presence of a complete immune system. Thus, we suggest that the main reason why the observation made in humans could not be confirmed in this mice study is the combination of a lack of an intact immune system in the Balb/c nu/nu mice and a relatively large tumour, probably with a lot of mature vessels.

References


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