ABSTRACT

Cutaneous melanoma is an immunogenic tumour that is often characterized by the presence of tumor infiltrating lymphocytes (TILs). The degree of such infiltration together with cell activation state are considered significant prognostic factor representing the immune reaction against the tumour. Patients with peri-tumoral infiltration may have better prognosis and may also achieve a better response to IL2 immunotherapy. Aim of this study was to evaluate $^{99m}$Tc-IL2 in vivo binding to cutaneous melanoma infiltrating lymphocytes and if such uptake correlates with immunological and histological data, thus providing useful prognostic information for IL2 therapy. There are evidences that the expression of CD25 is a good marker of tumor toxicity of T lymphocytes on melanoma cells. **Methods:** So far we studied 30 patients with cutaneous lesions suspect for melanoma. Planar gamma camera images over known tumour sites were acquired one hour after the injection of 3 mCi of $^{99m}$Tc-IL2. Peri-tumoral uptake of $^{99m}$Tc-IL2 was measured as Target/Background (T/B) radioactivity ratio. All patients underwent surgery and histological evaluation was performed. The percentage of different peripheral blood lymphocyte subsets (CD3, CD4, CD8, CD16, CD25) and the percentage of IL2R positive cells on tumour histological sections were also measured. **Results:** Results of this study showed that T/B ratios significantly correlated with the number of IL2R in TILs, while they do not correlate with the number of low affinity IL2R expressed on melanoma cells. **Conclusion:** $^{99m}$Tc-IL2 scintigraphy could allow to quantify in vivo the amount of IL2R+ve
cells infiltrating lesions, suggesting its use for prognostic purposes and to select patients who may benefit of IL2 immunotherapy.

Key words: Inteleukin-2, scintigraphy, melanoma, TIL.

INTRODUCTION

Melanoma is a common tumour of the skin with a frequent rate of metastases and poor prognosis. If the tumour is diagnosed at an early stage, when no metastases are present, surgical treatment is effective. It has been observed that most melanomas, either primitive or metastatic, are characterised by the presence of tumour infiltrating lymphocytes (TILs) and that this correlates with a better prognosis (1, 2).

The role of the immune reaction against melanoma is also confirmed by promising results obtained by the use of TILs as adjuvant therapy for stage III (regional lymph nodes) melanoma (3). On the basis of this observation, different immunomodulatory therapies have been developed during the past years, particularly those based on interleukin 2 (IL2) and Interferon-g. IL2 was shown to be a potent in vitro stimulator of the generation of cytotoxic T lymphocytes (CTL) and non-T-cell receptor restricted LAK cells (lymphokine-activated killer), both of which lyse in vitro tumour targets. Treatment with intravenous high-dose bolus IL2 has been shown to mediate objective responses in 15% of patients with metastatic melanoma (4). Approximately 8% of patients exhibit a complete response, which is durable in most cases (5).

IL2 treatment is characterised, however, by high costs and the frequent occurrence of side effects, among them the capillary-leak syndrome with its associated hypotension is the more severe, requiring inotropic support for patients during treatment. There is a great need for accurate prognostic indicators that can identify “a priori” those patients who are most likely to benefit from biological therapies. Several studies have attempted to identify predictive factors for IL2 response. In a study of 81 patients receiving various IL2 regimens, increased C-reactive protein levels and the presence of visceral metastases were found to be negatively related with response (6). One of the highest predictors of response is the presence of only subcutaneous and/or cutaneous metastases (7). There are evidences that the expression of CD25 is a good marker of tumor toxicity of T lymphocytes on melanoma cells (8).

Scintigraphy by $^{99m}$Tc-IL2 can detect in vivo the presence of activated organ infiltrating lymphocytes and in particular those in an activated state expressing the IL2 receptors (IL2R). Scintigraphy with both $^{125}$I- or $^{99m}$Tc-labelled IL2 has been extensively used to image chronic mediated inflammatory disorders (9, 10). Aim of this study was to evaluate whether $^{99m}$Tc-IL2 can be used to detect peri-tumoural infiltration by activated lymphocytes in patients with melanoma undergoing surgical treatment. If this approach proves to be successful the use of $^{99m}$Tc-IL2 in future might be envisaged for the selection of patients with metastatic diseases with peri-tumoural lymphocytic infiltration who are likely to respond to immune treatments and to evaluate their efficacy, also providing possible prognostic information on the disease outcome.
MATERIALS AND METHODS

Patients: 30 consecutive patients with cutaneous atypical pigmentary lesions, undergoing surgical resection of the lesion, were enrolled in the study. All lesions were analysed with respect to tumour invasiveness (Breslow depth, mm, and Clark level, I to V) and lymphocytic infiltration (extent of the infiltrate: 0 to 3, and type: absent, brisk non brisk, 0 to 2). The study was approved by the local Ethic Committee and all patients gave their written informed consent before entering the study. Peripheral lymphocyte subsets (CD3, CD4, CD8, CD16 and CD25) were also measured in all patients.

Evaluation of IL2R expression on melanoma lesions: The analysis of IL2R (CD25) expression on histological sections was performed in 17 out of 21 melanoma lesions due to technical problems. Five µm sections were deparaffinized and endogenous peroxidase activity was blocked with H2O2. Primary antibody anti-human IL2R (CD25), (dilution 1:200, neoMarkers, Fremont, CA, USA) was incubated for 1 hour at room temperature followed by a secondary antibody incubation (biotinylated goat anti-mouse, dilution 1:40) for 30 minutes at room temperature. Then, avidin biotin amplification (ABC kit, Dakocytomation, Glostrup, Denmark) was added for 30 minutes. Incubation with AEC (3-amino-9-ethylcarbazole) chromogen kit (Sigma, St. Louis, MO, USA) at room temperature for 5 to 10 minutes produced a red reaction pigment. Cell counting of CD25 positive was done at a magnification of 400X using a test grid with a 0.22 mm² area. An average of 20 fields per section or a number of microscopic fields until the SEM was <5% were counted (11).

99mTc-IL2 scintigraphy: All patients underwent 99mTc-IL2 scintigraphy. One hour after intravenous injection of 3 mCi of 99mTc-IL2, static images over known lesions were acquired. Accumulation of 99mTc-IL2 into the lesions was semi quantitatively calculated as target/background (T/B) radioactivity ratios. For this purpose, an irregular region of interest (ROI) was drawn over the lesion; an irregular ROI was drawn around the first ROI and the area between the two ROIs was considered as background.

Statistical analysis: Correlations between T/B ratios with immunological and tumoural parameters were calculated using Pearson coefficient for continuous variables and Spearman Rho coefficient for categorical variables. Mann-Whitney test was used to evaluate the presence of differences between means of groups. 99mTc-IL2 scintigraphy was considered positive if a significant uptake on tumour lesions were detected.

RESULTS

Histological features and expression of CD25 in melanomas and benign cutaneous lesions: Out of 30 patients with atypical pigmentary lesions, 21 were affected by malignant melanoma and 9 by other benign cutaneous lesions. Melanomas were classified as macular (n=11) or nodular lesions (n=10) accordingly to their size and/or their growth through the skin. 20 out of 21 melanoma lesions revealed a lymphocytic infiltration. An inverse correlation was observed between the degree of lymphocytic infiltration and the Clark level (r=-0.552, p=0.014). CD25 expression was detected both on TILs and on melanoma cells, but the intensity of staining was significantly lower in the latter. In details, a positivity to CD25 on melanoma cells was
present in 7 out of 17 melanoma studied lesions, with a variable expression (see table 1 for details). As far as the presence of CD25 expression on TIL is concerned, this was observed in 15 out of 17 lesions analysed, but also in this case the degree of IL2 receptors expression was considerably variable and ranging from 5 to 60% (table 1). The degree of CD25 expression was significantly higher in nodular as compared to macular melanomas (28.89±18.53% vs 8.75±11.26%; p=0.007 Mann-Whitney). No correlation was observed between the degree of CD25 expression on melanoma cells and the degree of lymphocytic infiltration.

**99mTc-IL2 scintigraphy:** 15 out of 21 (71.4 %) melanoma patients and 2 out of 9 patients with benign lesions (22.2%) showed a significant tumoral uptake of **99mTc-IL2** (Figures 1-6).

**Figure 1:** **99mTc-IL2** scintigraphy (A) in a patient affected by a large melanoma of the left gluteus (B). Despite the large size of the tumor the distribution of radioactivity is non homogeneous reflecting the patchy infiltration by activated T-lymphocytes. CD25 staining showed a mild positivity on neoplastic cells and a strong diffuse positivity on lymphocytes (C).

**Figure 2:** **99mTc-IL2** scintigraphy (A) in a patient affected by a small melanoma near the right clavicle (B). Despite the small size of the tumor (green arrow), regional lymph nodes are also detectable (red arrow), reflecting the active immune response inside primary and metastatic tumor. CD25 staining (C) showed a mild positivity in neoplastic cells and a strong diffuse positivity on lymphocytes.

**Figure 3:** **99mTc-IL2** scintigraphy in a patient affected by a melanoma of the right foot. A moderate **99mTc-IL2** uptake is detectable in the tumor area (arrow).
Figure 4: $^{99m}$Tc-IL2 scintigraphy in a patient affected by a melanoma of the left foot. A strong $^{99m}$Tc-IL2 uptake is detectable in the tumor area (arrow).

Figure 5: $^{99m}$Tc-IL2 scintigraphy in a patient affected by a melanoma of the left arm. No significant $^{99m}$Tc-IL2 uptake is detectable in the tumor area (arrow).

Figure 6: $^{99m}$Tc-IL2 scintigraphy in a patient affected by an angioma of the left thigh. No significant $^{99m}$Tc-IL2 uptake is detectable in the tumor area (arrow).
All patient positive to $^{99m}$Tc-IL2 scintigraphy, a lymphocytic infiltration was observed. Although CD25 was not tested in benign lesions, the two benign lesions (atypical nevi) positive to $^{99m}$Tc-IL2 scintigraphy, also showed a significant lymphocytic infiltration at histology. The degree of $^{99m}$Tc-IL2 uptake on melanoma lesions, as assessed by T/B ratios, ranged from 1 to 1.81.

**Correlations between scintigraphic findings and histological features:**

T/B ratios calculated on scintigraphic images strongly correlate to the degree of CD25 on TIL ($r=0.811$, $p=0.0001$; figure 7a). Accordingly to CD25 evaluation on histology, $^{99m}$Tc-IL2 scintigraphy was truly positive in 10 out of 14 lesions (Sensitivity 71.4), but in 3 out 4 the degree of CD25 expression was below 15%. All CD25-ve lesions revealed a negative scan (Specificity 100%). Surprisingly, no correlation was found between T/B ratios and CD25 expression on melanoma cells (Figure 7b). Results of T/B ratios also correlated with the “severity” of lymphocytic infiltration ($r=0.454$, $p=0.039$), although the lesions classified as brisk were only 3.

**DISCUSSION**

Results described herein showed that $^{99m}$Tc-IL2 scintigraphy is able to image TILs expressing IL2 receptors (IL2R) and that the degree of $^{99m}$Tc-IL2 uptake is correlated with the percentage of IL2R positive cells. Moreover, despite T/B ratios are not considerably high, all positive lesions could be easily detected on gamma camera imaging (see figures 1-4). In details, whilst a strong correlation was described between the degree of CD25 expression on TILs with $^{99m}$Tc-IL2 uptake expressed as T/B ratio, scintigraphic findings did not correlate at all with the expression of CD25 on melanoma cells. In addition, when partial a correlation procedure (computing partial linear coefficients between T/B ratios and the expression of CD25 on TILs while controlling for the effects of CD25 on melanoma cells) was applied, results of linear correlation were similar ($r=0.809; p=0.0001$), thus confirming the negligible
effect of neoplastic cells CD25 expression on $^{99m}$Tc-IL2 uptake. A possible explanation to these results could be due to the antibody used for the detection of IL2 receptor positive cells, although the lower density of CD25 expression on melanoma cells (as detected on histological studies) should be considered. As a matter of facts, in vivo uptake of radiolabelled IL2 mainly represents its binding on high affinity IL2 receptors, which are trimers composed by the alpha, beta and gamma chains. The antibody used for immunohistochemical studies recognizes only the alpha chain of this receptor, which alone characterizes the low-affinity receptors for IL2. While the alpha chain of the IL2 receptor is expressed in a high percentage of tumour cells, the beta chain could also be detected, although in a limited number of cases (12). High affinity IL2 receptors are indeed rare on melanoma cells. On the other hand, TILs are equipped with a fully functional IL2 receptor system, thus explaining TILs activation and expansion during immunotherapy with IL2 (13). High affinity IL2R expression on TILs represent a marker of lymphocyte activation, which occur only when lymphocytes recognize specific melanoma antigens, that are classified as tumor-associated testis-specific antigens, melanocyte differentiation antigens, and mutated or aberrantly expressed antigens (14). IL2 immunotherapy enhances tumour aggression by T lymphocytes, mainly stimulating those lymphocytes expressing high affinity receptors. In this view, the degree of IL2 expression on TILs could represent a more objective marker of lymphocyte strength against tumours as compared to the absolute number of TILs present in the lesion or their infiltrating pattern. Nevertheless, a positive trend was observed between T/B ratios calculated and the severity of lymphocytic infiltration, although the number of lesions classified as Brisk is limited (n=3). As previously described, the presence of a brisk pattern has a strong predictive value for primary cutaneous melanomas with a vertical growth phase (15). $^{99m}$Tc-IL2 scintigraphy could be therefore used in patients with stage III/IV melanomas candidates for immunotherapy. The presence/absence of IL2R positive cells on melanoma positive lymph nodes or distant metastases could provide useful prognostic information on the possible effect of immunotherapy. Finally, significant correlations were reported between the degree of $^{99m}$Tc-IL2 uptake with the number of CD16, mainly Natural Killer cells (positive correlation) and with the absolute number of circulating lymphocytes (CD3+ve, inverse correlation) or with the CD4 population (inverse correlation). Interpretation of such correlations is difficult to analyse. Nevertheless, CD16 expressed on human NK cells represent signal-transducing molecules that, upon ligand binding, induce surface activation molecules (like the IL2R) and cytokines relevant to NK cell biology and functions (16). It can be speculated that the correlation between IL2 receptor expression on TILs could be influenced by the number of circulating CD16+ve cells.

**CONCLUSION**

Results reported suggest a possible role of $^{99m}$Tc-IL2 scintigraphy in patients affected by cutaneous melanoma. In particular, this technique could add precious information on tumoral biological behaviour possibly addressing therapeutic interventions. In order to test the prognostic role of $^{99m}$Tc-IL2 scintigraphy a longitudinal study is mandatory, by correlating the results of
immunotherapy with IL2 accordingly with scintigraphic findings obtained before.

REFERENCES


## TABLE 1
Immunological, histological and scintigraphic data in patients with melanoma

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*Extent of perilesional lymphocytic infiltration
† Brisk=2, Non Brisk=1, Absent=0 indicate the severity of lymphocytic infiltration (15)
Alessandro, 2002, “Tramonto”