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Editorial

Detection of Early Micrometastases in Malignant Melanoma

E.M. Rankin

Departments of Immunology and Medical Oncology, The Netherlands Cancer Institute, Antoni van Leeuwenhoek Huis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

MELANOMA, ONCE it has metastasised beyond the locoregional lymph nodes, is associated with a dismal prognosis with a median survival of at best 7.5 months [1]. Despite all the advances in management, prognosis has remained unaltered during the last quarter of a century. The incidence is rising rapidly, doubling every 10 years. It is forecast that the overall mortality from malignant melanoma will peak early in the next century [2–5].

Even patients with locoregional spread to the lymph nodes do badly, 70% progressing to die eventually of systemic disease. Until recently, no form of intervention has been shown to materially affect the outcome in stage III disease. The experience with adjuvant interferon alpha-2b administered at the maximum tolerated dose is encouraging with an improvement in disease-free and median survival in the treated group of approximately 1 year [6]. Even so, only a small number of patients receiving this toxic treatment actually benefit from it.

Recent experience with non-toxic treatments, e.g. vaccination with the Mage 1 or 3 peptide in patients with advanced disease [7], suggests there is hope for a patient-tailored approach to adjuvant immunotherapy using a cocktail of peptides in those with early stages of melanoma at high risk of relapse. We need to identify patients with truly microscopic metastases since optimal immunomodulation at this stage of disease is likely to result in a significantly increased cure rate. Indeed, the impact of adjuvant interferon alpha is greatest in those with clinically inapparent microscopic lymph node involvement [6] supporting the idea that the optimal setting for adjuvant therapy is in those with minimal residual disease.

Clinically, negative lymph nodes in patients with thick primaries $\geq$ 4 mm have a 60% chance of harbouring microscopic nodal metastases. However, elective lymph node dissection in patients with high risk primary lesions has failed to show any survival advantage in randomised prospective studies [8–10]. Sentinel node mapping with selective lymph node dissection has a greater than 95% predictive value of the nodal status in those with tumours $\geq$ 1 mm thick [11]. It is hypothesised that if the sentinel node is negative, the remainder of the lymph nodes in the basin would be negative, so that such patients could be spared the morbidity of a complete nodal dissection. Whether elective nodal dissection in those with a positive sentinel lymph node leads to improved survival is the subject of an ongoing international randomised trial.

The likelihood with which lymph node spread is discovered depends on the diligence with which it is sought, the number of nodes identified and the methods used to examine them. Routine histological examination of lymph nodes enables approximately one abnormal cell to be identified in a background of 10^4 normal cells, but if only one or two sections are cut from the centre of each node then less than one thousandth of the submitted tissue is examined in detail. This leads to an underestimation of the number of involved nodes. Serial sectioning with immunohistochemical staining, e.g. with antibodies to S-100 protein or HMB 45 antigen, will increase the sensitivity of detection to approximately one abnormal cell per 10^5 background normal cells, but is not routinely conducted because of constraints on time and expense.

The development of sensitive assays which combine reverse transcription with the polymerase chain reaction (PCR) for the detection of messenger RNA of tyrosinase means we now have a highly sensitive specific tool for the detection of microscopic tumour. This technique has been used for the identification of tumour spread to the lymph nodes [12], to the circulation [13–17] and as reported in this issue (pages 1664–1667), to the subcutaneous fat around the primary lesion [18].

The gene for tyrosinase is tissue specific and is found in melanocytes and malignant melanoma cells. Tyrosinase is a key enzyme during melanin synthesis. Importantly, tyrosinase may be recognised by cytotoxic T cells, and two different T cell epitopes have been identified, one in the context of HLA-A2 [19] and the other in the context of HLA-A24 [20]. This means that immunotherapeutic approaches involving tyrosinase peptides or proteins could be used as adjuvant therapy in patients with micrometastatic spread of tyrosinase-containing tumour.

Detection of tumour in lymph nodes using molecular pathological techniques greatly enhances the ability to diagnose microscopic locoregional tumour spread. Using PCR to identify cells containing mutations of *TP53* or *K-RAS*, Hayashi and associates showed that the presence of genetically detectable tumour cells in histologically negative lymph nodes from

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patients with colon cancer was strongly associated with clinical outcome [21]. 27 of 37 patients with PCR-positive lymph nodes developed a recurrence within 5 years, whereas all 34 patients with negative nodes remained disease-free. One of the drawbacks of this approach is that fragments of dying or dead tumour containing the sequence of interest may be transported to the regional nodes where detection may be misinterpreted as indicating the presence of live neoplastic cells. Tumour heterogeneity is also a problem especially if the target being sought is restricted to a few tumour subclones. It is relevant to note that tyrosinase expression within a tumour is usually homogenous, unlike the heterogeneous pattern of expression of other melanoma-associated antigens such as gp100 [22] or Mage 3 (our own observations) seen within tumours for a single patient.

Wang and colleagues studied regional lymph nodes electively removed from patients with clinical stage I or II melanoma [13]. Nodes were examined both histologically following routine procedures and with an RT–PCR method. Nineteen of the lymph node preparations (containing an average of five nodes per patient) from 29 patients were positive for tyrosinase including all eleven which were histologically positive. Further analysis of the RT–PCR-positive, histologically-negative nodes using serial sections and immunohistochemical stains still failed to identify malignant cells. Experiments to determine sensitivity of the RT–PCR method showed a lower limit of detection of approximately three melanoma cells per 10^7 nodal lymphocytes. It would be interesting to know how many of the histologically-negative sentinel nodes in patients who subsequently relapse locally actually contain tumour identifiable by RT–PCR. There is an urgent need for more studies of this technique to be incorporated into prospective studies so that the clinical significance of the findings of occult metastases in lymph nodes in melanoma can be determined.

An area of increasing interest has been the identification of circulating malignant cells in the peripheral blood. Most studies have used RT–PCR with nested primers for tyrosinase originally described by Smith and associates [13–17, 22]. This method can detect one melanoma cell in 2 ml normal blood [13], or one melanoma cell in 10^6 peripheral blood lymphocytes [16]. Attempts have been made to give a semiquantitative assessment [15] or a truly quantitative estimate of the number of circulating cells. One of the difficulties of quantification is that there may be interpatient variation in the level of expression of tyrosinase in the melanoma cells. If reliable means of quantifying cells were available, it would allow an estimate of the relative numbers of circulating cells in the patient and possibly permit early detection of progression or regression in the same way as more conventional markers e.g. CEA or CA-125 may mimic the behaviour of cancer. Clearly, precautionary measures need to be taken during the assay to prevent false-positive results due to contamination with PCR products or genomic DNA, and controls need to be built in for the RNA isolation, cDNA synthesis and the PCR steps.

If a combination of markers is used to provide multiple targets in the PCR assay, e.g. tyrosinase, Mage 3 and p97, then the chance of identifying circulating cells increases [16]. Knowledge of the characteristics of the primary tumour would help to identify which markers should be used for follow-up of circulating cells in the individual patient. However, it should be remembered that some markers, e.g. Mage 3, are more commonly expressed in metastases than in the primary tumour.

The chance of finding cells in the circulation depends on the site from which the blood sample is taken. The number of viable cells in the circulation falls by at least two orders of magnitude during the first pass through a capillary bed. Thus, to detect tumour spill during resection, intra-operative sampling of peripheral venous blood from the antecubital fossa is likely to give a much lower yield than direct sampling of the venous blood draining from the tumour, e.g. sampling of the proximal subclavian vein during breast cancer surgery [23] or the portal vein following colon cancer resections [24].

Circulating tumour cells have been found at all stages of disease and, not surprisingly, are more common in those with manifest tumour. The short- or long-term significance of the presence of tumour cells in the blood is unclear. The prognostic significance of systemic dissemination of single cells has been demonstrated in some patients with breast or gastrointestinal cancer [25]. However, the finding that 80% of patients undergoing radical prostatectomy for prostate cancer had circulating prostate cancer cells following operation is far higher than previously thought, and far exceeds the expected recurrence rate [26]. Denis and colleagues identified circulating melanoma cells after lymph node dissection in 7 of 16 patients [27]. These cleared after 4 weeks and only one patient has relapsed. In contrast, Battayani and associates found that 5 of 8 patients with circulating cells prior to removal of involved lymph nodes relapsed within 6 months, whereas only one of 10 PCR-negative patients relapsed in the same time period [28]. Thus, one must question whether circulating tumour cells are always capable of further metastasising and developing their own supportive vasculature.

One further refinement of the attempts to identify early occult disease is to look at the site of resection of the primary disease. Proebstle and colleagues examined portions of subcutaneous fat in tissue below the primary melanoma (pages 1664–1667) [18]. Random inoculation of known amounts of cells from a tyrosinase positive cell line enabled calculation that the lower limit of detection with RT–PCR of tumour cells in subcutaneous fat was between 10^2 and 10^4 cells. The enzymatic fat dissociation step prior to preparation of the subcutaneous tissue for PCR probably leads to loss of melanoma cells resulting in the lower sensitivity of this assay compared with that for circulating tumour cells. It is unclear in the paper by Proebstle and colleagues in how many cases the tissues from patients were examined at the time of the removal of the primary when contamination with normal melanocytes was unlikely and in how many at the time of secondary wide excision following an earlier resection, when the chance of contamination of subcutaneous fat would be higher. In 4 of the 10 patients examined, tyrosinase transcripts were found in the subcutaneous fat, in 3 of 5 Clark IV and in only 1 of 5 Clark III lesions. The prognostic significance of these findings is unclear, but the study is worth extending to a larger group of patients. If melanoma cells are reproducibly and frequently found in the local fat, one wonders why local recurrence is so rare. Are these cells simply harbingers of the fact that cells have already spread elsewhere, e.g. via lymphatic or blood vessels? Are they more susceptible to immune surveillance than cells in the circulation? Are they even capable of further cell division?

We are entering an era where detailed and sensitive studies can be made to identify the presence of occult metastases at

various sites. These approaches should be incorporated into some of the large adjuvant studies with melanoma which are due to start shortly. In this way, we can assess the prognostic significance of the finding of minuscule amounts of occult metastases. Perhaps in several years time we will have sufficient data to know whether we should alter therapy according to whether micrometastases have been identified or not.

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