Activated Stat-3 is detected in melanoma but not in benign melanocytic lesions.

Activated Stat-3 in Melanoma

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Background: Recent studies have demonstrated that the Src-Stat pathway may play an important role in melanoma. We examined the expression of phosphorylated Stat-3 (pStat-3), activated Stat-1 (pStat-1) and interferon alpha receptor subunit 1 (IFNAR-1) in human melanocytic neoplasms.

Methods: Compound nevi (6), dysplastic nevi (4), congenital nevi (2), primary melanoma (14), and sentinel lymph node metastasis (40) were examined. Specimens were evaluated for phospho-Stat-1 (pStat-1), phospho-Stat-3 (pStat-3), and IFNAR-1 by immunohistochemistry. Staining was scored from 1 to 3 based on a composite score that took into account both the percentage of tumor cells staining and the intensity of stained cells.

Results: Normal melanocytes or benign nevi expressed little pStat-1, pStat-3, or IFNAR-1. In primary cutaneous melanoma, 6 of 14 skin biopsies showed activated Stat-3. However, in melanoma metastatic to regional lymph nodes, 16 of 26 had activated Stat-3 but only 6 of 23 had activated Stat-1. Melanoma tumors had high levels of either pStat-3 or pStat-1 but not both. All melanoma specimens but not benign melanocytes had cytoplasmic IFNAR-1 staining. An increase in Stat-3 activity was seen in melanoma but not in benign nevi or skin melanocytes. There appeared to be an inverse correlation between the levels of pStat-3 and pStat-1 in a given specimen.

Conclusions: The relationship between activated Stat-3 and biological behavior of melanocytic lesions observed in this study warrants further exploration.

Introduction

The Stat transcription factors play an important role in transducing signals from growth factors and other stimuli to the nucleus where they activate transcription from target genes. Src signaling is transmitted in part through activation and DNA binding of pStat-3 as well as through other downstream pathways. Interferon alpha-2b (IFN-α2b), which is widely used for treating melanoma, also signals through the Stat pathway. IFN-α2b clearly exerts direct antiproliferative effects on melanoma cells, and IFN-α2b-resistant cell lines show deficient tyrosine phosphorylation and downstream signaling or overexpression of the inhibitory SOCS-1.
There is also evidence that IFN-α2b exerts its effects through Stat signaling in the immune system rather than directly. In a series of experiments, Lesinski et al. showed that IFN effects are abrogated in a Stat-1 deficient mouse. Interestingly, the antitumor effects of IFN were preserved in mice injected with Stat-1-deficient melanoma, suggesting that the Stat-1 level in the host immune system was more important than the Stat-1 level in the melanoma. There appeared to be a lack of correlation between Stat-1 and Stat-2 levels and clinical outcome in 28 melanoma patients treated with adjuvant IFN-α2b. Our studies have shown that Stat-3 activation is present in a large proportion of melanoma cell lines and human metastatic melanoma tumors. In these studies, Stat-3 activity was determined by electrophoretic mobility shift assays, which did not identify the cell type with activated Stat-3 in tumor tissues. Disruption of Stat-3 signaling by introduction of a dominant negative Stat-3 into B16 mouse melanoma cells induced cell death. Blockade of Src signaling by a small molecule inhibitor also led to melanoma cell apoptosis. Introduction of a dominant negative Stat-3 plasmid by electroporation into pre-existing B16 melanoma in mice caused inhibition of tumor growth and regression of tumors. In addition, a potent bystander effect was seen indicating that inhibition of Stat-3 signaling interrupted paracrine signaling within the tumor microenvironment. Blockade of Src signaling by a small molecule inhibitor produced the same effect.

While these in vitro and animal models are valuable, the levels of activated Stat-3 and other components of this pathway in human melanocytic lesions are not known. This review defines these components in a variety of melanocytic neoplasms and in skin melanocytes.

Methods

Patients and Pathologic Specimens

All patients in this study were treated at Moffitt Cancer Center between 1990 and 2003. A tissue acquisition protocol was approved by the Institutional Review Board (IRB) at the University of South Florida (MCC 12897). Patient demographic information was determined by chart review. Patient samples included normal skin, benign melanocytic lesions, primary cutaneous melanoma, mucosal melanoma, and metastatic melanoma, including sentinel lymph nodes with metastatic melanoma. Primary cutaneous melanomas ranged from in situ to invasive lesions with a depth of 7.43 mm. Lymph nodes were obtained from 40 patients with a diagnosis of malignant melanoma metastatic to sentinel nodes and treated with postoperative IFN-α2b between 1992 and 2002. One representative positive sentinel lymph node paraffin block from each patient was selected, and slides were cut from this block and stained for phosphorylated Stat (pStat) and IFN-α2b.
The 40 patients with metastatic melanoma ranged in age from 16 to 74 years. The male:female ratio was 2.4:1. The distribution of primary melanomas was as follows: 11 axial, 6 head and neck, 5 extremity, and 2 acral lentiginous. The median tumor thickness was 3.68 mm (range 1 to 10 mm).

Fourteen of the 40 sentinel nodes stained had no tumor remaining in the block. This is due to the fact that the majority of sentinel lymph node tumor deposits were micrometastatic disease (<2 mm), and thus the tumor was exhausted in additional sections performed for immunostaining. No evidence of staining for pStat-3 was seen in normal lymphocytes within the sentinel or nonsentinel lymph nodes (Fig 4). Similar to findings in the skin, there was staining of pStat-3 in blood vessels within the lymph nodes, in melanoma cells, and interestingly also in dendritic cells within the lymph nodes. IFN-α2b receptor expression was also seen in melanoma cells within lymph nodes (Fig 5).

Staining for pStat-3 showed the greatest variation among the 26 evaluable cases, in both the percentage of tumor cells staining and the intensity of the staining. Some tumors stained uniformly for this marker; in others, staining was confined to limited areas of the tumor, while others were negative, similar to the pattern seen in primary melanoma. The staining was predominantly

**Immunohistochemistry for pStat-1, pStat-3, and IFN-α2b Receptor**

Mouse monoclonal antibody to IFNAR-1 was obtained from Research Diagnostics Inc (Pleasant Hill, New Jersey). Rabbit polyclonal antiphospho Stat-1 and Stat-3 (anti-phospho tyrosine 705-Stat-3 [Tyr705] and antiphospho tyrosine 705-Stat-1 antibodies) were obtained from Cell Signaling Technologies, Beverly, Massachusetts. Anti-rabbit IgG from goat antiserum was obtained from Sigma-Aldrich Co, St. Louis, Missouri; 5-μm sections were cut from formalin-fixed, paraffin-embedded tissues and pretreated with microwave and citrate/trypsin antigen retrieval for Stat staining. No antigen retrieval was used for IFNAR-1 staining. The Stat-1 and Stat-3 stained slides were incubated with the primary antibody at a 1:300 dilution overnight at 4° C. The IFNAR-1 slides were stained for 60 minutes with primary antibody at a dilution of 1:500. Vectastain kits were used for amplifying antibody responses (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, California) on a Dako Autostainer Universal Staining System (Dako North America, Inc, Carpinteria, California) using DAB or NovaRED chromogen (Vector Laboratories). Slides were counterstained with modified Mayer's hematoxylin, dehydrated through ascending grades of ethanol, cleared with xylene, and mounted with resinous mounting medium. Rabbit IgG was used as the negative control reagent.

Staining of tumor cells for pStat-3 was scored by a formula calculating both the percentage of stained cells and the intensity of staining as follows: 0 = no staining, 1 = 1% to 25%, 2 = 26% to 50%, 3 = more than 50% cells stained, and intensity = 0–3. The sum of these was the composite Stat-3 score: 0 (0), 1 (1–2), 2 (3–4), and 3 (5–6). For pStat-1 and IFNAR-1 staining, a score of 0 to 3 was assigned based on the intensity of cellular staining.

**Results**

Normal skin showed no appreciable staining for either pStat-1 or IFN-α2b receptor (Fig 1A). Normal skin stained with pStat-3 showed positivity in dermal dendrocytes, adnexal structures, and the epidermis but not in melanocytes (Fig 1B). There was no significant staining of IFN-α2b, pStat-1, or pStat-3 in any of the melanocytic nevi examined (representative example of IFN-α2b receptor and pStat-3 (Fig 2). One of the compound nevi showed focal staining of melanocytes for pStat-3 immediately below an area of ulceration. Six of the 14 skin biopsies of malignant melanoma showed nuclear staining for pStat-3 (Fig 3). The presence of pStat-3 staining demonstrated no significant relationship to several histologic prognostic parameters, including thickness and ulceration (data not shown).
nuclear, and cases with the most intense staining always demonstrated specific nuclear staining. The 26 patients were followed for a mean interval of 30 months (range 2 to 70 months). During this time, 11 patients recurred and 8 patients died. Of the 16 patients with high-grade staining (level 3 or 2), 9 patients (56%) had recurrences, and of the 10 patients with low-grade staining (level 0 or 1), 3 patients had recurrences (30%). Of 8 patients who died during the course of this study, 6 (75%) had high- or moderate-grade pStat-3; only 2 patients (25%) had low or absent pStat-3.

Among 40 lymph node samples, 23 had detectable tumor. Seventeen of these 23 patients had low levels or absent Stat-1 staining, 8 of whom (47%) had recurrences. Of the 6 patients with high-level Stat-1 staining, only 2 (33%) had recurrences. The distribution of staining for Stat-1, Stat-3, and IFN-α2b receptor is shown in the Table. Interestingly, almost every melanoma examined had either high pStat-3 or high pStat-1 but not both. Staining for IFN-α2b receptor was more uniform, although a few cases showed diffuse, intense staining (Fig 5).

**Discussion**

IFN-α2b is widely used to treat patients with melanoma. Until the current study, however, it was unclear if IFN-α2b receptors were actually expressed in human melanoma in vivo. Our results demonstrate that all tumors examined did express the IFN-α2b receptor. While many studies have shown the presence of signaling downstream of the IFN-α2b receptor in melanoma cells, it has not been clear if these are present in benign melanocytes and in benign melanocytic nevi or only in malignant melanoma. Similarly, while there is a wealth of data on pStat-3 in melanoma cell lines and melanoma tumor preparations, there has not been a systematic analysis of pStat-3 levels in human melanocytic neoplasms.

Several melanoma cell lines and metastatic melanoma tumor samples show activation of Stat-3. Blockade of Stat-3 via a dominant negative Stat-3 construct or blockade of the upstream Src tyrosine kinase induces apoptosis in a melanoma cell line. Blocking Stat-3 activation can inhibit both vascular endothelial...
growth factor (VEGF) and hypoxia-inducible factor 1α (HIF-1α). Stat-3 can also lead to suppression of the innate and adaptive immune response. Cumulatively, these data suggest that Src and Stat-3 may play a role in melanoma progression and/or development. These results are especially interesting in light of the current development of specific Src inhibitors, some of which are currently in early-phase clinical trials in both solid tumor and hematologic malignancies.

We sought to extend these observations to human melanocytic lesions. Since these neoplasms encompass a spectrum ranging from benign to malignant, they can be useful in delineating the locus and role of an aberrant pathway. Therefore, we examined by immunohistochemistry pStat-1, pStat-3, and the IFN-α2b receptor.

There was no pStat-3 in cutaneous melanocytes (the precursor cells of all melanocytic lesions) in normal skin, while hair follicles, dermal dendrocytes, and blood vessels did show positivity for pStat-3. The normal epidermis also showed no staining for IFN-α2b receptor or pStat-1. Multiple types of benign melanocytic neoplasms including junctional, compound, congenital, and dysplastic nevi showed no significant pStat-3 or pStat-1. One exception was a compound nevus that showed focal pStat-3 positivity underlying an area of ulceration, likely related to activation of Stat-3 during the repair process. In contrast, frequent pStat-3 staining was found in cutaneous melanoma, thus suggesting that pStat-3 is associated with melanoma progression or development.

We also examined pStat-3 levels in a group of melanoma patients with sentinel lymph node positive melanoma. Of the 26 patients with significant pStat-3 levels, there was a 56% recurrence rate, while the recurrence rate was 33% in patients with low pStat-3. While strong pStat-1 was less frequent overall, there was a lower recurrence rate in these patients compared to those with weak or absent pStat-1 (33% vs 47%). Similarly, 29% of patients with high levels of IFN-α2b receptors recurred, while 56% of patients with low-level expression of IFN-α2b receptors recurred. Further analysis by log rank testing for time to recurrence for pStat-3 levels did not show the differences to be statistically significant (P = .19), although this type of analy-

Fig 4A-B. — Sections of a sentinel lymph node immunostained for pStat-3 and counterstained with hematoxylin. Photomicrographs A and B show the sections at 200× and 400× magnification. (A) A blood vessel (BV) is indicated by the arrows as being positive for pStat-3. (B) Melanoma cells (Mel) show the typical nuclear staining for pStat-3 while lymphocytes are completely negative. Also shown is the dendritic cell (DC), which is also positive for pStat-3.

Fig 5A-B. — Sections of a lymph node containing metastatic melanoma cells examined by immunohistochemistry for IFN-α2b receptor and counterstained with hematoxylin. Note the diffuse cytoplasmic staining of IFN-α2b receptor in melanoma. Photomicrograph A, 200× magnification; B, 400× magnification.
sis lacks power, and probably only large magnitude differences could be uncovered in a small sample such as this. In this regard, a recent study noted that pretreatment Stat-1 and Stat-2 levels did not correlate with outcome in patients treated with IFN-α2b. In this study, 28 melanoma tumors, both primary and metastatic, were examined, but the activated or pStat species was not studied and the specimens were not examined for pStat-3 or IFNAR-1. We are currently in the process of acquiring a larger set of specimens to definitively answer this question.

Our findings are also provocative with regard to the development of Src and Stat inhibitors. As shown by the signal transduction inhibitor trastuzumab in HER2-positive tumors and epidermal growth factor receptor (EGFR) inhibitors with respect to EGFR mutations, the selection of patients based on target enrichment is critical. Following the same logic, our data provide a method for selecting melanoma tumors with enhanced pStat-3 for therapeutic trials with inhibitors of the Src-Stat pathway. We are currently investigating the relationship of phosphorylated Src levels to that of phosphorylated pStat-3 for therapeutic trials with inhibitors of the Src-Stat pathway.

Disclosures
No significant relationship exists between the authors and the companies/organizations whose products or services may be referenced in this article.

Research support for this study was provided by Schering-Plough to Dr Daud.

References

Table. — Lymph Node Metastatic Melanoma Stained and Quantified for IFN-α2b Receptor, pStat-1, and pStat-3*

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* Recurrence and death were established by chart review and from the melanoma database at Moffitt Cancer Center.